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Modulation of Chemical Carcinogen-Induced Unscheduled DNA Synthesis by Dehydroepiandrosterone (DHEA) in the Primary Rat Hepatocytes

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Modulation of unscheduled DNA synthesis by dehydroepiandrosterone (DHEA) after exposure to various chemical carcinogens was investigated in the primary rat hepatocytes. Unscheduled DNA synthesis was induced by treatment of such direct acting carcinogens as methyl methanesulfonate (MMS) and ethyl methanesulfonate (EMS) or procarcinogens including benzo(a)pyrene (BaP) and 7,12-dimethylbenz(a)anthracene (DMBA). Unscheduled DNA synthesis was determined by measuring [methyl-^{3}H]thymidine radioactivity incorporated into nuclear DNA of hepatocytes treated with carcinogens in the presence or absence of DHEA. Hydroxyurea (5×10^{-3} M) was added to growth medium to selectively suppress normal replication. DHEA at concentrations ranging from 1×10^{-6} M to 5×10^{-4} M did not significantly inhibit unscheduled DNA synthesis induced by either MMS (1×10^{-4} M) or EMS (1×10^{-2} M). In contrast, DHEA significantly inhibited unscheduled DNA synthesis induced by BaP (6.5×10^{-5} M) and DMBA (2×10^{-5} M). DHEA-induced hepatotoxicity in rats was examined using lactate dehydrogenase (LDH) release as an indicator of cytotoxicity. DHEA exhibit no significant increase in LDH release compared with the solvent control at 18 h. These data suggest that nontoxic concentration of DHEA does not affect the DNA excision repair process, but it probably influence the enzymatic system responsible for the metabolic activation of procarcinogens and thereby decreases the amount of the effective DNA adducts formed by the ultimate reactive carcinogenic species.

Key words : Dehydroepiandrosterone, Unscheduled DNA synthesis, Primary rat hepatocytes

INTRODUCTION

The endogenous steroid, dehydroepiandrosterone (DHEA) was first isolated from human urine in 1934 (Butenandt and Dannenbaum, 1934). The levels of DHEA and its sulfate derivative rise in early life to reach a maximum at about 25 years of age, and then decline profoundly throughout adult life (Migeon *et al.*, 1957; Orentreich *et al.*, 1984). These steroids originate largely from the adrenal cortex and are markedly elevated in certain adrenal tumors or by the administration of corticotropin (ACTH) (Vande Wiele *et al.*, 1963; Baulieu *et al.*, 1965). Studies with rodents have demonstrated that low levels of DHEA and DHEA-sulfate are associated with the development of age-related diseases, such as cancer or atherosclerosis (Gordon *et al.*, 1987; Orner *et al.*, 1996). Administration of DHEA was reported to protect laboratory rodents against spontaneous and carcinogen-induced tumors (Nyce *et al.*, 1984; Lubet *et al.*, 1998). It was demonstrated that DHEA inhibited the binding of 7,12-dimethylbenz(a)anthracene (DMBA) to DNA *in vivo* (Prasanna *et al.*, 1986) and the growth of mammary carcinoma *via* the androgen receptor (Gatto *et al.*, 1998).

Substantial evidence from *in vivo* and *in vitro* experiments indicates that at least some of afore mentioned anticancer effects of DHEA are mediated through inhibition of the pentose phosphate pathway at the glucose-6-phosphate dehydrogenase step (Oertel and Benes, 1972; Levy, 1979). Therefore, the inhibitory effect

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of DHEA on cancer development may be due to the restriction on the availability of NADPH and 5-carbon sugar which are generated in the pentose phosphate pathway. DHEA and a related hormone, 16-bromoepiandrosterone were reported to inhibit the generation of superoxide anion radical from 12-*O*-tetradecanoylphorbol-13-acetate(TPA)-stimulated human polymorphonuclear leukocytes (Whitcomb and Schwartz, 1985). Since superoxide has been implicated in tumor promotion, this inhibition identifies a possible mechanism whereby these steroids inhibit tumor formation (Kensler and Trush, 1984). Recent study demonstrated that the treatment of p53-/-animals with DHEA resulted in decrease of *bcl*-2 but not *bax* mRNA levels in the thymus. This finding suggested that DHEA modulated tumorigenesis through alterations in the apoptotic pathways (Wang *et al.*, 1997). The mechanistic aspects of the physiological effects by DHEA have been extensively investigated and well documented.

In the present study, we investigated the effects of DHEA on the unscheduled DNA synthesis induced by various chemical carcinogens in the primary rat hepatocytes. The results of this work suggest an involvement of DHEA in the metabolic activation step of chemical carcinogens.

MATERIALS AND METHODS

Chemicals

DHEA, hydroxyurea, bovine serum albumin, DMBA and benzo(a)pyrene (BaP) were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Methyl methanesulfonate (MMS) and ethyl methanesulfonate (EMS) were obtained from Aldrich Chemical Co. (Metuchen, NJ, U.S.A.). [Methyl-^{3}H]thymidine was purchased from Amersham/Searle Co. (Arlington Heights, IL, U.S.A.). Other chemicals used were of the highest analytical quality.

Preparation of isolated rat hepatocytes

Hepatocytes were obtained from male Sprague-Dawley rats *via* an *in situ*collagenase perfusion procedure (Oldham *et al.*, 1980). Cell viability was greater than 80% as measured by trypan blue exclusion. Monolayer hepatocyte cultures were established by plating 3 ml of a cell suspension containing 1×10^6 hepatocytes /ml in William's E Medium (WE, Gibco, Grand Island, NY, U.S.A.) containing 17% fetal bovine serum (FBS) into a collagen coated tissue culture dish (60 × 15 mm). The hepatocytes were allowed to attach to the culture dish for 4 h at 37°C in a humidified 95% air/ 5% CO_2 atmosphere. A medium change was routinely performed 4 h after plating to remove unattached cells.

Treatment of hepatocytes with test compounds

After attachment, the hepatocytes were refed with WE containing 10% FBS and an appropriate concentration of test chemical, and incubated for 18 hours at 37°C in a humidified 95% air/ 5% CO_2 atmosphere. Solvent concentrations in the medium did not exceed 1%. Each treatment group was plated in triplicate. In all instances, controls were exposed to the same volume of dimethylsufoxide (DMSO). Cytotoxicity induced by test chemicals was examined by quantitative measurement of LDH leakage into the cell medium using a commercial LDH assay kit (Boehringer Mannheim, Mannheim, Germany: Pounds *et al.*, 1982).

Measurement of unscheduled DNA synthesis

Unscheduled DNA synthesis, following treatment of hepatocytes in culture with chemical carcinogens and DHEA, was measured by determining the amount of [methyl-^{3}H]thymidine (3 μ Ci/plate) incorporated into nuclear DNA in the presence of 5 mM hydroxyurea. At the end of experiment, the monolayers were washed with ice-cold 20% trichloroacetic acid (TCA) solution.The cells were scraped off by means of a rubber policeman. The lysate was collected by 3 washes of cold 10% TCA solution. The precipitate was sedimented at 2000 × g for 10 min and hydrolyzed in 0.5 N perchloric acid. After incubation at 90°C for 20 min, aliquots of the hydrolyzed DNA fraction (500 μl) were counted in scintillation vials containing 10 ml of liquid scintillation fluid. DNA content was determined from the hydrolysate by use of diphenylamine reagent. Calf thymus DNA was used as a standard for DNA quantitation. All data were analyzed by one-way analysis of variance and Bonferroni's modified *t*-test and a significance level of $p<0.05$ or $p<0.01$ was used to evaluate differences between two groups (Wallenstein *et al.*, 1980).

RESULTS

Chemical carcinogen-induced hepatocytotoxicity was studied *in vitro* using lactate dehydrogenase (LDH) release as an indicator of cytotoxicity. Primary rat hepatocytes were incubated with various chemical carcinogens (1×10^{-4} M MMS, 1×10^{-2} M EMS, 6.5×10^{-5} M BaP, 2×10^{-5} M DMBA) for 24 h. Samples of the culture medium were taken at 5, 18 and 24 h post-treatment and assayed for the presence of LDH activity. The results showed that chemical carcinogens did not cause a significant increase in LDH release for an incubation time upto 24 h at the concentrations used in the present study (data not shown). Therefore, an incubation time was chosen as 18 h in the subsequent experiments.

LDH release induced by DHEA was measured in the presence of chemical carcinogen (1×10^{-4} M MMS) and shown in Fig. 1. DHEA did not cause a biologically

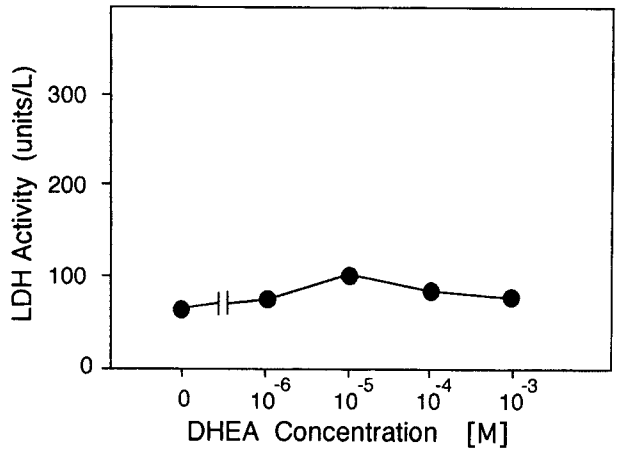

Fig. 1. Effect of DHEA on LDH release from isolated rat hepatocytes. Primary rat hepatocytes were obtained as described in "Materials and Methods", incubated with various concentrations of DHEA and MMS ($1\,5\,10^{-4}$ M) for 18 h and the cytotoxicity was measured using a LDH assay kit. The data are shown as the mean ± standard deviation of three separate experiments.

significant increase in LDH release at concentrations ranging from 1×10^{-6} M to 1×10^{-3} M, whereas dramatic increase in the LDH release was observed at concentrations higher than 1×10^{-3} M. This result indicates that DHEA did not elicit an appreciable cytotoxicity at the concentration range (1×10^{-6} M~5×10^{-4} M) used in the present study. Similar results were obtained after incubation with DHEA and other chemical carcinogens including EMS, BaP and DMBA (data not shown).

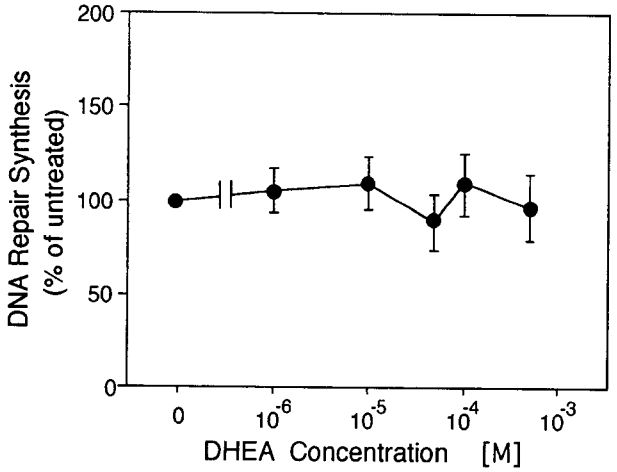

Fig. 2. Effect of DHEA on unscheduled DNA synthesis in isolated rat hepatocytes. Unscheduled DNA synthesis was measured by determining the amount of [methyl-^{3}H]thymidine incorporated into nuclear DNA. The data are normalized against the [methyl-^{3}H]thymidine incorporation in control(vehicle-treated) cultures. The data are shown as the mean ± standard deviation of three separate experiments.

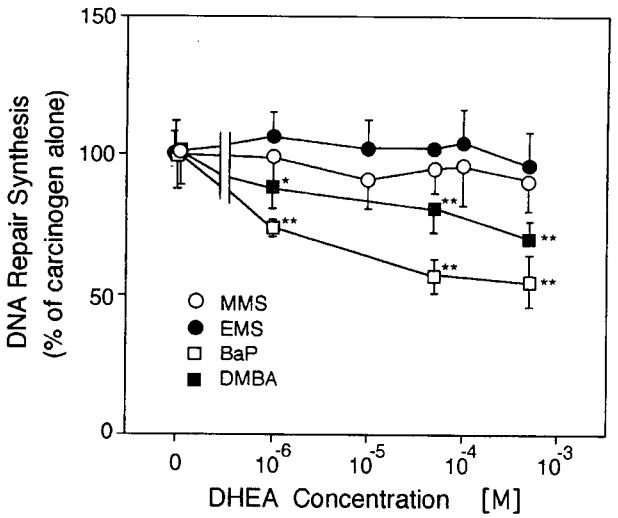

Fig. 3. Effect of DHEA on unscheduled DNA synthesis induced by direct acting carcinogens (MMS or EMS) or procarcinogens (BaP or DMBA). Primary rat hepatocytes were incubated with various concentrations of DHEA in the pre-sence of chemical carcinogens ($1\,5\,10^{-4}$ M MMS, $1\,5\,10^{-2}$ M EMS, $6.5\,5\,10^{-5}$ M BaP or $2\,5\,10^{-5}$ M DMBA) and unscheduled DNA synthesis was measured. The data are normalized against the [methyl-^{3}H]thymidine incorporation in cultures treated with chemical carcinogen alone. The data are shown as the mean ± standard deviation of three separate experiments.

The effect of DHEA on unscheduled DNA synthesis was studied to assess the possible genotoxicity induced by this compound itself. DHEA did not cause a significant increase in unscheduled DNA synthesis when compared with the DMSO control at all concentrations tested (Fig. 2).

The effect of DHEA on chemical carcinogen-induced unscheduled DNA synthesis was examined. Primary rat liver cell cultures were exposed to various concentrations of DHEA in the presence of direct acting carcinogens (1×10^{-4} M MMS, 1×10^{-2} M EMS, Fig. 3) or procarcinogens (6.5×10^{-5} M BaP, 2×10^{-5} M DMBA, Fig. 4). As shown in Fig. 3, addition of DHEA did not affect repaired DNA synthesis induced by direct-acting carcinogens (MMS or EMS). On the other hand, DHEA decreased repaired DNA synthesis induced by BaP or DMBA in a dose-dependent manner (Fig. 4). These data suggest that DHEA may alter or probably inhibit the metabolic activation of procarcinogens to their ultimate reactive carcinogenic species.

DISCUSSION

Rat liver possesses the enzyme systems for metabolizing a wide array of chemical carcinogens (procarcinogens) known to require metabolic activation (Weisburger and Williams, 1975). The reaction of chemical carcinogens with DNA in this system is not limited by the

stability of metabolites or their ability to penetrate into cells. Thus, rat liver cells are the ideal culture system to screen suspected chemical carcinogens (Williams, 1975). Repaired DNA synthesis after DNA damage has been studied since the mid-1960s (Rasmussen and Painter, 1964). Unscheduled DNA synthesis occurs to repair DNA damage in non-S phase cells after carcinogen treatment. DNA damage induced by chemical carcinogens can be classified into three main groups: base modification, strand breaks and cross-links. Excision repair, which consists of excision of the adducts, DNA-strand polymerization and ligation, can repair most forms of base damage. Strand breaks and cross-links may also be repaired by excision repair. Misrepair or incomplete repair may be directly related to the initiation step of carcinogenesis. Most chemical carcinogens are strong electrophiles which react with DNA resulting in the repaired DNA synthesis.

We used unscheduled DNA synthesis as a marker of DNA damage in hepatocytes after treatment of chemical carcinogens. In the present study, the repaired DNA synthesis was measured by determining the incorporation of [methyl-^{3}H]thymidine into DNA. Hydroxyurea (5×10^{-3} M) was added in growth medium to increase the sensitivity of the assay by selectively suppressing normal DNA replication. The inability of DHEA to elicit DNA repair as measured by unscheduled DNA synthesis (shown in Fig. 2) implies that this compound does not cause DNA damage *in vitro*. However, the lack of unscheduled DNA synthesis induction does not completely preclude the possibility of DNA damage. If a compound produced DNA adducts which were not subject to excision repair, it would not be detected as a genotoxin in unscheduled DNA synthesis assay.

Our data clearly showed that DHEA was not able to inhibit the genotoxicity induced by direct acting carcinogens, while it inhibited the genotoxicity induced by procarcinogens which require metabolic activation. These contrasting results suggest that DHEA may either decrease or alter activation of procarcinogens to their ultimate carcinogenic species.

LDH release, an indicator of cytotoxicity in cultured primary hepatocytes, was measured after treatment with DHEA. DHEA did not elicit an appreciable cytotoxicity as measured by LDH release at the highest concentration tested. This indicates that DHEA does not affect a critical step in DNA excision repair *via* a cytotoxic mechanism.

Several reports demonstrate that DHEA can alter the metabolism of procarcinogens. Prasanna *et al.* (1989) have shown that DHEA inhibited the binding of DMBA to hepatic DNA *in vivo* in spite of the increased metabolic activation of the carcinogen due to increased detoxification. One possible approach to decide whether or not DHEA inhibits carcinogen-induced unscheduled DNA synthesis by modulating the metabolism of this hydrocarbon to a reactive electrophile would be to determine if DHEA can modulate the genotoxicity of the synthesized carcinogenic metabolites of procarcinogens.

In conclusion, the present study demonstrated that non-toxic concentration of DHEA does not affect DNA excision repair process, but it may affect the enzymatic system for the metabolic activation of procarcinogens, and thereby decrease the amount of the effective DNA adducts formed by the ultimate reactive carcinogenic species.

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