Dehydroepiandrosterone supplement increases malate dehydrogenase activity and decreases NADPH-dependent antioxidant enzyme activity in rat hepatocellular carcinogenesis

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Abstract
Beneficial effects of dehydroepiandrosterone (DHEA) supplement on age-associated chronic diseases such as cancer, cardiovascular disease, insulin resistance and diabetes, have been reported. However, its mechanism of action in hepatocellular carcinoma in vivo has not been investigated in detail. We have previously shown that during hepatocellular carcinogenesis, DHEA treatment decreases formation of preneoplastic glutathione S-transferase placental form-positive foci in the liver and has antioxidant effects. Here we aimed to determine the mechanism of actions of DHEA, in comparison to vitamin E, in a chemically-induced hepatocellular carcinoma model in rats. Sprague-Dawley rats were administered with control diet without a carcinogen, diets with 1.5% vitamin E, 0.5% DHEA and both of the compounds with a carcinogen for 6 weeks. The doses were previously reported to have anti-cancer effects in animals without known toxicities. With DHEA treatment, cytosolic malate dehydrogenase activities were significantly increased by ~5 fold and glucose 6-phosphate dehydrogenase activities were decreased by ~25% compared to carcinogen treated group. Activities of Se-glutathione peroxidase in the cytosol was decreased significantly with DHEA treatment, confirming its antioxidative effect. However, liver microsomal cytochrome P-450 content and NADPH-dependent cytochrome P-450 reductase activities were not altered with DHEA treatment. Vitamin E treatment decreased cytosolic Se-glutathione peroxidase activities in accordance with our previous reports. However, vitamin E did not alter glucose 6-phosphate dehydrogenase or malate dehydrogenase activities. Our results suggest that DHEA may have decreased tumor nodule formation and reduced lipid peroxidation as previously reported, possibly by increasing the production of NADPH, a reducing equivalent for NADPH-dependent antioxidant enzymes. DHEA treatment tended to reduce glucose 6-phosphate dehydrogenase activities, which may have resulted in limited supply for de novo synthesis of DNA via inhibiting the hexose monophosphate pathway. Although both DHEA and vitamin E effectively reduced preneoplastic foci in this model, they seemed to function in different mechanisms. In conclusion, DHEA may be used to reduce hepatocellular carcinoma growth by targeting NADPH synthesis, cell proliferation and anti-oxidant enzyme activities during tumor growth.

Key Words: Hepatocellular carcinoma, DHEA, malate dehydrogenase, NADPH and glucose 6-phosphate dehydrogenase

Introduction
DHEA and DHEA-sulfate are the precursors of androgens and estrogens produced from adrenal glands (Roberge et al., 2007). Their levels start to decrease after middle age and animal and human studies have reported that age-associated chronic diseases involving dysregulation of metabolism may be due to the decline in the levels of these compounds (Labrie, 2007). Although there have been in vivo studies about the protective effects of DHEA against cancer, obesity, diabetes, postmenopausal osteoporosis, sexual dysfunction and cardiovascular disease as modulators of adrenocortical steroid synthesis (Allolio et al., 2007; Kim and Choi, 2005), its mechanism of action needs to be studied more in detail before safely used as supplements in humans for different diseases.

Reactive oxygen species are continuously formed as by-products of aerobic metabolism and from reactions of drugs and environmental toxins such as carcinogens (Cerutti, 1985). These play important roles in tumor progression by damaging DNA, proteins and unsaturated lipids (Champe & Harvey, 1987). The cells have protective mechanisms that can minimize the toxic effects from these compounds. These mechanisms include antioxidant chemicals (e.g. vitamin E), enzymes that catalyze antioxidant reactions using NADPH as a source of reducing electrons and a liver microsomal cytochrome P-450 mono-oxygenase system, which also utilizes NADPH to convert steroids and drugs to soluble forms (Wu et al., 1989).

We have previously shown that DHEA decreases glutathione
S-transferase placental from (GST-p) foci in the liver and lipid peroxidation using a hepatocellular carcinoma model in rats (Kim & Choi, 2005). Here, we aimed to determine the mechanism of protective effects of DHEA supplement during chemical hepatocellular carcinogenesis in vivo in comparison to those with vitamin E supplement, an effective intracellular reducing agent.

Materials and Methods

Animals

Six-week-old male Sprague Dawley rats were purchased from Seoul National University and housed at the animal care facility at Seoul National University (Seoul, Korea). All rats were kept under standard temperature, humidity, and timed lighting conditions and were provided with rat chow and water ad libitum. Animals were induced with hepatocellular carcinoma by i.p. injection of diethylnitrosamine (200 mg/kg body weight) followed by administration of diets containing 0.01% of 2-acetylaminofluorene for 6 weeks. Partial hepatectomy surgeries were performed 1 week after the start of 2-acetylaminofluorene diet to effectively induce progression of tumor as previously published (Ito et al., 1988).

Diets and materials

Rat chow was purchased from Purina (purified rodent diet 5053, St. Louis, Missouri) and was supplemented with 1.5% vitamin E (Sigma T-3376, DL-α tocopheryl acetate, St. Louis, MO) and 0.5% DHEA (Sigma D-400, DHEA 3-acetate, St. Louis, MO) as described previously (Kim & Choi, 2005).

Biochemical assays

Fresh or frozen livers were weighed and five volumes of ice-cold homogenization buffer [154 mM KCl, 50 mM Tris-HCl, 1mM EDTA, pH 7.5] were added. The tissue was homogenized and was fractionated by spinning at 1,000 g for 13 min at 4°C. The middle layer was centrifugated at 10,000 g for 13 min at 4°C. Then the supernatant was centrifugated at 100,000 g for 65 min at 4°C to obtain cytosolic and microsomal fractions as described in detail previously (Kim & Choi, 2005).

Malate dehydrogenase activities

Malate dehydrogenase activities were measured in the cytosolic fraction using modified methods of Ochoa (1969). Briefly, 500 μl of triethanolamine buffer [0.4 M, pH 7.4], 50μl of L-malate [30 mM], 100 μl of MnCl₂, 4H₂O [0.12 M] and 200μl of NADP [3.4 mM] were mixed with 1,400 μl of cytosolic fraction using a vortex. Absorption at 270 nm during 1 min at 26°C was measured every 6 seconds. One unit is increased in absorption by 0.01 during 1 min.

Glucose 6-phosphate dehydrogenase activities

Glucose 6-phosphate dehydrogenase activities were measured in the cytosolic fraction using modified methods of Lohr and Waller (1974). Briefly, 2.4 ml of triethanolamine buffer [50 mM, pH 7.5] and 0.5 ml of cytosolic fraction (~0.8 mg/ml protein) were mixed using a vortex. Then, 50 μl of NADP solution was added and was kept at 25°C for 5 minutes. Next, 50 μl of glucose 6-phosphate was added and increases in absorption was immediately measured at 340 nm for every 2 minutes up to 6 minutes. Specific activity was calculated as μmole of substrate converted per minute per mg protein.

Se-glutathione peroxidase

Cytosolic Se-glutathione peroxidase activities were measured using a modified method of Tappel (1978) as previously described.

Microsomal cytochrome P-450

Hepatic microsomal fraction was used to measure cytochrome P-450 content (Omura & Sato, 1964). Fresh microsomal fraction was diluted with phosphate buffer (pH 7.4) to contain ~ 1 mg/ml protein and sodium dithionate was added and was saturated with CO gas for 1 minute. Then, absorptions of reduced carbon monoxide at 450 and 490 nm were determined spectrophotometrically. Molar extinction coefficient used was 91/mM/cm.

Protein concentration

Protein concentrations were determined by the Bradford assay using the Biorad Protein assay reagent (Biorad, Hercules, CA).

Statistical analysis

Data are expressed as mean ± SE. SAS software was used for Duncan’s multiple range test and one-way ANOVA to determine statistical differences (p<0.05).

Results

DHEA treatment increased cytosolic malate dehydrogenase activities

We previously showed (Kim & Choi, 2005) that DHEA treatment decreased NADPH-utilizing antioxidant enzymes in this model so we first determined if activities of enzymes that regulate NADPH synthesis were altered. Interestingly, DHEA
DHEA increases malic enzyme activity in vivo

Fig. 1. DHEA treatment increases cytosolic malate dehydrogenase activities. The level of cytosolic malate dehydrogenase activity was determined by biochemical enzymatic assay. Hepatocellular carcinoma was induced with diethylnitrosamine, 2-acetylaminofluorene and ~70% partial hepatectomy using male Sprague-Dawley rats. The animals were treated with control diet without a carcinogen, carcinogen diets with 1.5% vitamin E, 0.5% dehydroepiandrosterone (DHEA) and both 1.5% vitamin E and 0.5% DHEA. After ultracentrifugation of freshly-obtained liver, cytosolic fraction was obtained and used to measure malate dehydrogenase activities. Activities were normalized against protein concentration. One-way ANOVA test was used to determine significance (n=6~9 each). Means with different subscripts are significantly different at \( p < 0.05 \) by Duncan's multiple range test.

Activities of cytosolic glucose 6-phosphate dehydrogenase had a tendency to decrease with DHEA treatment

Although DHEA treatment increased malate dehydrogenase activities, it had a strong tendency to decrease glucose 6-phosphate dehydrogenase activities (Fig. 2). Glucose 6-phosphate dehydrogenase activity was increased by almost 1.5 fold with the carcinogen treatment and DHEA supplement decreased it to the level of those of non-carcinogen treated group (COO vs. TOO). However, in the combination group (TED), the enzyme activity was not suppressed as seen in DHEA-only treated animals. a, b, c; one-way ANOVA, \( p<0.05 \).

DHEA treatment decreased hepatic cytosolic Se-glutathione peroxidase activities.

Activities of cytosolic Se-glutathione peroxidase activities tended to increase with carcinogen treatment (COO vs. TOO). The enzyme activities were inhibited almost 50-60% with DHEA treatment compared to controls (COO and TOO vs. TOD). Vitamin E treatment also decreased the activities significantly and a synergistic inhibitory effects were seen in combination treatment group (TOO vs. TED).

Hepatic microsomal cytochrome P-450 content was not altered with DHEA treatment.

To examine the mechanism of action of DHEA on hepatic microsomal cytochrome P-450 mono-oxygenase system, cytochrome P-450 content was measured. DHEA treatment did not affect cytochrome P-450 content (Fig. 4). We also measured microsomal NADPH cytochrome P-450 reductase activities but they were also not affected with DHEA treatment (data not shown).

Discussion

Here we studied the mechanism of action of DHEA during hepatocellular carcinogenesis in vivo. We found that DHEA significantly increased activities of malate dehydrogenase, an enzyme important for supplying biochemical reductant NADPH in the cytosol. On the other hand, glucose 6-phosphate dehydrogenase activities were reduced by ~25% with DHEA treatment possibly due to the high level of NADPH/NADP⁺ ratio in the cytosol, provided by increased malate dehydrogenase activity. Reports showed that DHEA treatment decreased mRNA levels of hepatic malate dehydrogenase in broiler chickens (Zhao et al., 2007) and increased the activity in non-insulin dependent diabetic rats (Ladriere et al., 1997). However, mRNA levels do not always correlate with levels of enzyme activity and the dose, duration and the disease model used in these studies were different from the current study. Marrero, et al. showed that DHEA treatment in female mice increased hepatic malate
dehydrogenase activities by 2-3 fold (Marrero et al., 1990) but did not alter glucose 6-phosphate dehydrogenase activity, similar to the data from this study. Further studies using different organ systems in various diseases models are needed to clarify effect of DHEA on these NADP-dependent enzymes.

Under most metabolic conditions, the ratio of NADPH/NADP⁺ is high enough to inhibit glucose 6-phosphate dehydrogenase activity. However, with increased demand for NADPH in the cells (e.g. toxic and oxidative stress), the ratio of NADPH/NADP⁺ decreases and flux through the hexose monophosphate pathway is enhanced by increasing the activity of glucose 6-phosphate dehydrogenase (Champe & Harvey, 1987). Glucose 6-phosphate dehydrogenase is the key enzyme regulating the first and irreversible step of the pentose phosphate pathway. This pathway can then provide ribose-5-phosphates, a precursor for DNA synthesis in cell proliferation. During promotional phase of carcinogenesis, the demand for ribose-5-phosphate for de novo synthesis of DNA may be increased and it is thus particularly important that DHEA treatment suppressed this rate limiting enzyme of the pentose phosphate pathway, glucose 6-phosphate dehydrogenase. This may be one of the protective mechanisms of DHEA during carcinogenesis as reported previously (Kim & Choi, 2005). Although malate dehydrogenase activities increased with DHEA treatment in this study, it has been reported that its activity is ~15% of that of glucose 6-phosphate dehydrogenase in rat adrenal cortex (Frederiks et al., 1990). If this is the case in rat liver, the decrease in glucose 6-phosphate dehydrogenase would surpass the relative change in the malate dehydrogenase activity in the inhibition of pentose phosphate pathway.

Increases in malate dehydrogenase activities by DHEA treatment may have also provided NADPH to reduce glutathione efficiently. This antioxidant effects of DHEA was seen in decreased Se-dependent glutathione peroxidase activities (Fig. 3), confirming less oxidative damage with DHEA treatment as previously reported (Kim & Choi, 2005). Reduced glutathione (a tripeptide-thiol) can detoxify hydrogen peroxide in most cells in a NADPH-dependent manner (Frederiks et al., 1990) catalyzed by glutathione reductase. Although the cells were producing higher amounts of NADPH through malate dehydrogenase, it may be that the cells were not triggered to increase NADPH-dependent antioxidant enzymes due to already decreased oxidative stress level in the cell by direct or indirect antioxidant effect of DHEA. The possible mechanism of the non-enzymatic antioxidative activity of DHEA needs further studies in the future. Furthermore, the effects of DHEA may depend on the phase in which these enzymatic activities were measured during the progression of hepatocellular carcinoma.

Vitamin E has been known to be a strong nonenzymic antioxidant, preventing oxidative damage in the cell components by reducing free radicals as reported (Muller et al., 2003). Here, vitamin E decreased glutathione peroxidase activities (Fig. 3) probably through different mechanism than that from DHEA; vitamin E did not alter malate dehydrogenase or glucose 6-phosphate dehydrogenase activities in this study.

Lastly, the content of cytochrome P-450 (Fig. 4) and NADPH-dependent cytochrome P-450 reductase activities (data not shown) were not affected by DHEA treatment. These data suggest that the mechanism of actions of DHEA is independent from these liver microsomal cytochrome P-450 mono-oxygenase system, a major pathway for the hydroxylation of aromatic and aliphatic compounds such as stroids and alcohols and carcinogens (Wu et al., 1989) which also uses NADPH to to convert them into soluble forms. This is different from previous reports that showed that DHEA decreased cytochrome P-450 enzymes, especially P4501A and 3A (Fitzpatrick et al., 2001). It is possible that because we measured total levels of cytochrome P-450 in this study, we may not have found any difference in the overall levels and may have missed isozyme-specific effects of DHEA.

Our data suggest that DHEA has protective effects during hepatocellular carcinoma by upregulating NADPH-producing...
DHEA increases malic enzyme activity \textit{in vivo}.

Malate dehydrogenase and may thus spare cells from reactive oxygen species-induced damages during carcinogenesis. In the future, more studies with different concentration of DHEA in various stages of cancer progression will be required to find out further details of its mechanism of action \textit{in vivo}.

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