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Research Article

The in vitro antiapoptotic effect of dehydroepiandrosterone sulfate in mouse thymocytes and its relation to caspase-3/caspase-6

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Abstract. The effects of dehydroepiandrosterone sulfate (DHEAS) on thymocyte apoptosis induced by dexamethasone (DEX) were investigated. Apoptosis was measured by using agarose gel electrophoresis of DNA, the terminal deoxynucleotidyltransferase (TdT)-mediated dUTP nick end labeling (TUNEL) assay and flow cytometry. Our results showed that preincubation with 1×10^{-4} M DHEAS protected thymocytes from DEX-induced apoptosis in vitro. Moreover, we found no blocking effect on the DEX-induced activation of caspase-3 and caspase-6 by the preincubation of thymocytes with DHEAS. This may be interpreted to mean that the antagonism of DHEAS to DEX-induced apoptosis is not related to the activation of these downstream caspases which play a critical role in the execution of apoptosis.

Key words. Dehydroepiandrosterone sulfate; apoptosis; thymocytes; dexamethasone; caspase-3; caspase-6.

Dehydroepiandrosterone (DHEA, 5-androsten-3β-ol-17-one) and its sulfate ester (DHEAS) are the most abundant adrenal steroids in human blood circulation [1]. DHEA is formed from its precursor cholesterol and pregnenolone [2] and sulfated to DHEA sulfate (DHEAS), the inactive form, by DHEA-sulfotransferase before entering circulation [3]. In the periphery, DHEAS is hydrolyzed to DHEA by steroid sulfatase (SS) [4]. DHEA was first known as precursor of sex hormones and has been found to have many diverse beneficial effects in various types of cells, tissues and organs. DHEA was reported to be associated with general better health, disease protection and antiaging effects and thus earned the name 'fountain of youth' [5]. In humans, plasma levels of DHEA were found to increase during childhood and puberty, then decline with advancing age [6]. Moreover, substantially lower levels of DHEA were found to be accompanied with serious illness, including hypercholesterolemia, cancers, osteoporosis, human immunodeficiency virus (HIV) infection and other pathophysiological conditions [1].

Despite its global biological effects, there is little information on the cellular and molecular mechanism of action of DHEA. However, numerious evidence suggests that many biological effects of DHEA appear to oppose those of glucocorticoids [7]. The effect of glucocorticoids on the immune system, including thymus involution and thymocyte killing, is well established. The induction of thymocyte apoptosis by glucocorticoids was first reported by Wyllie in 1980 [8]. A decade later, May et al. reported that glucocorticoid-induced thymic involution and thymocyte lysis in mice was significantly blocked by DHEA pretreatment [6]. Our own results suggest that protection by DHEA against

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thymic involution and accordingly the weakening of the cell-mediated immune response may be associated with a wide range of beneficial effects from disease protection to delaying aging. We found that preincubation of thymocytes with DHEAS at 1×10^{-4} M attenuated apoptosis. Our study presented comprehensive, detailed and convincing evidence for the antiapoptotic effect of DHEA in thymocytes. Furthermore, we found that the inhibitory effect of DHEA on DEX-induced apoptosis is not via the blockage of caspase-3 or caspase-6 activation induced by this glucocorticoid.

Materials and methods

Animals. Male BALB/c mice (2–3 weeks old) were used for all experiments.

Preparation of thymocyte suspensions. Thymus glands were isolated, gently homogenized, transferred to PRMI1640 medium (pH 7.2) and then filtered through a nylon net (300 mesh). Cell suspension was diluted to 1.0×10^7 cells/ml and incubated at 37 °C in 5% CO_2 air.

DHEAS preparation. DHEAS was dissolved in alcohol and added to the culture medium to reach the desired concentrations. DHEAS solutions were adjusted to make an equal final concentration of alcohol (0.1%) in all treatments. The same amount of alcohol was added to culture medium as a vehicle control.

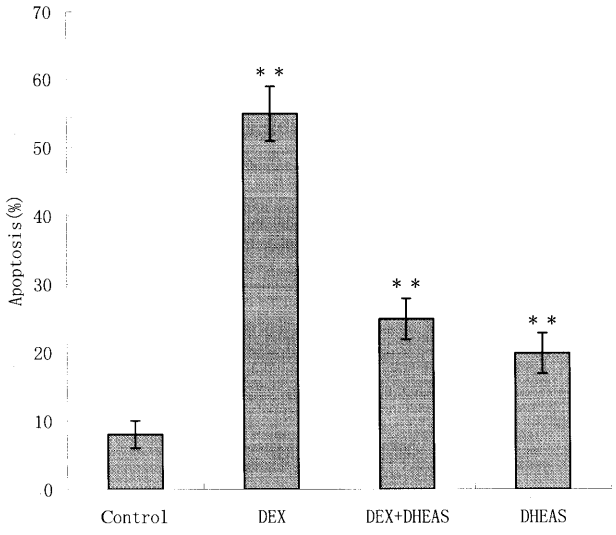

Figure 1. Percentage of apoptosis measured by flow cytometric assay. Values are the means of three separate experiments. Error bars indicate standard deviations. The level of significance was (**) $P < 0.01$ (Fisher's protected least-significant-difference, PLC). DEX, 1×10^{-6} M, DHEAS, 1×10^{-4} M.

Chemical treatments. The standard procedure of DHEAS + DEX treatment was as follows: DHEAS was added to cell suspensions and preincubated for 1.5 h. After that DEX was added to the mixture at a final concentration of 1×10^6 M. Cells were cultured for another 3 h. When the DHEAS alone was used in the treatment, the steroid was added to cell suspensions and then incubated for 4.5 h. In the treatment where only DEX was used, cells were incubated with medium containing 0.1% alcohol for 1.5 h before DEX was added and then incubated for 3 h. The working concentration of DEX is 1×10^6 M.

Agarose gel electrophoresis of DNA. DNA was extracted and separated by electrophoresis as previously described [9].

The TUNEL assay. Cells were brought to a slide and immobilized by polylysine. The TUNEL assay which detects DNA 3′-OH nicks in situ was performed according to manufacturer's (Boehringer Mannheim) instructions.

Flow cytometric analysis. Cells were analyzed by flow cytometry as described by Krishan [10] using a Coulter Elite Flow Cytometer. Data were treated using the Multicycle software program.

Detection of caspase-3 and caspase-6 activity. Activity of caspase-3 was measured as recommended by the manufacturer (Calbiochem). The substrates used are Ac-DEVD-pNA for caspase-3 and Ac-VEID-pNA for caspase-6. The protein concentration was measured according to Bradford's method [11].

Results

Antagonistic effect of DHEA on apoptosis induction by DEX as shown by flow cytometric analysis. Flow cytometric analysis is widely used in quantifying the apoptotic cells. When nucleus DNA content is lower than that at the G_0/G_1 phase, the cell is determined to be apoptotic. As shown in figure 1, DEX-induced apoptosis was markedly reduced by DHEA pretreatment. The data demonstrated that the percentage of apoptotic cells in DEX, DHEAS + DEX and DHEAS treatments were 55, 25 and 20%, respectively, indicating that DHEA blocked approximately 50% of thymocyte apoptosis. It is worthy of note that DHEAS alone also caused some cell death. The results are consistent with other reports [12]. The basis underlying this phenomenon is unknown.

Inhibition of DEX-induced DNA strand breaks in thymocytes by DHEAS pretreatment as detected by the TUNEL procedure. The TUNEL assay enables in situ detection of nuclear DNA strand breaks via visualizing DNA 3′-OH nicks. Our results showed that when thymocytes were treated with DEX (1×10^{-6} M), DEX (1×10^{-6} M) + DHEAS (1×10^{-4} M) and DHEAS

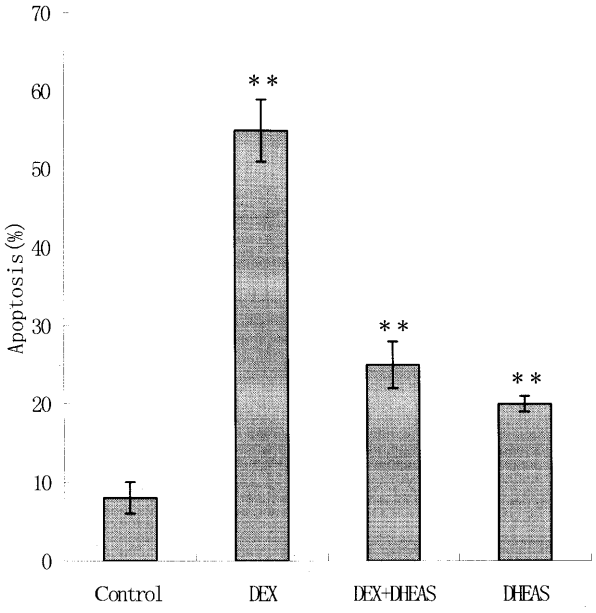

Figure 2. Percentage of apoptosis as detected by the TUNEL assay. Values are the means of three separate experiments. Error bars indicate standard deviations. The level of significance was (**) $P < 0.01$ (Fisher's protected least-significant-difference, PLC). DEX, 1×10^{-6} M, DHEAS, 1×10^{-4} M.

(1×10^{-4} M), the percentage of TUNEL-positive cells was 40, 20 and 15%, respectively (figs 2 and 3). When the concentration of DHEAS was lower than 1×10^{-4} M, no clear reduction in percentage of apoptotic cells was observed if compared with the treatment of DEX alone (data not shown). It appears that a concentration of 1×10^{-4} M, which is considered to be a physiological concentration, is required for DHEA and DHEAS to reverse the DEX-induced DNA stand breaks.

Prevention of DNA laddering by DHEAS treatment in DEX-treated thymocytes. DNA laddering, resulting from DNA degradation in to oligonucleosomal fragments by activated endonuclease during apoptosis, is a hallmark of apoptosis. Our study revealed that DNA from thymocytes treated with 1×10^{-6} M DEX alone showed clear ladders (fig. 4a). In the DHEAS + DEX treatment, when DHEAS concentration was below 1×10^{-4} M, DNA ladders were also obvious. If the concentration of DHEAS reached 1×10^{-4} M, DNA ladders were indistinct (fig. 4b). In addition, DHEAS alone at 1×10^{-4} M caused only faint ladders (fig. 4d). The results demonstrated a remarkable preventive effect on apoptotic DNA fragmentation by DHEA.

Effect of DHEA on DEX-induced activation of caspase-3 in thymocytes. DEX was found to activate caspase-3 in various cell types including rat thymocytes [13]. As shown in figure 5, the activation of caspase-3 was also observed in DEX-treated mouse thymocytes. But this activation was not affected by DHEAS pretreatment. The results implied that the antiapoptotic effect of DHEA in DEX-treated thymocytes was not through interfering with the caspase-3 activation.

Effect of DHEA on DEX-induced activation of caspase-6 in thymocytes. In addition to caspase-3, other caspases also play an important role during execution of apoptosis. In the case of mouse thymocytes, lamin B_1 degradation, which is catalyzed by caspase-6, was reported to precede DNA fragmentation during apoptosis [14]. It is thus reasonable to investigate whether caspase-6 activation is affected by DHEA. Our study shows that during DEX-induced thymic apoptosis, caspase-6 is activated. However, this activation was not impacted by DHEAS pretreatment (fig. 6). Thus the antiapoptotic effect of DHEA in DEX-treated thymocytes also does not appear to be due to blocking of caspase-6 activation.

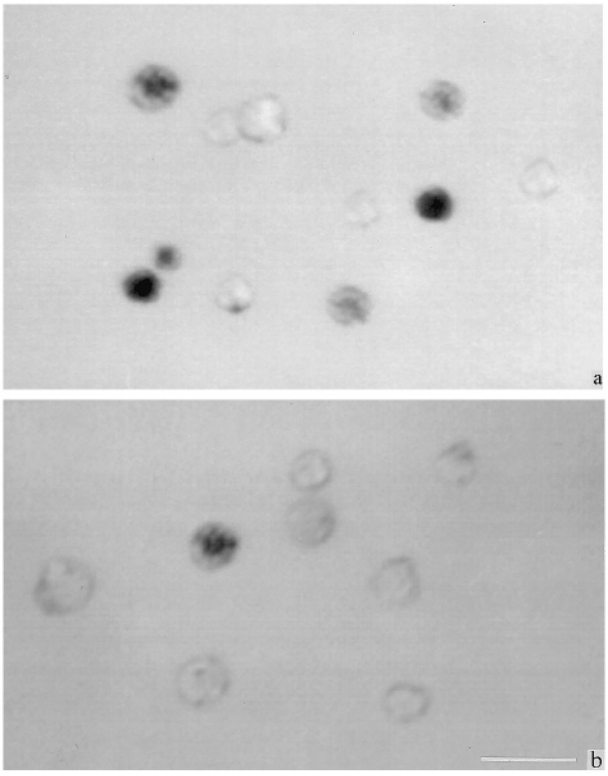

Figure 3. In situ detection of DNA fragments by the TUNEL assay. Bar, 10 μm. (*a*) DEX, 1×10^{-6} M; (*b*) DEX (1×10^{-6} M) + DHEAS (1×10^{-4} M).

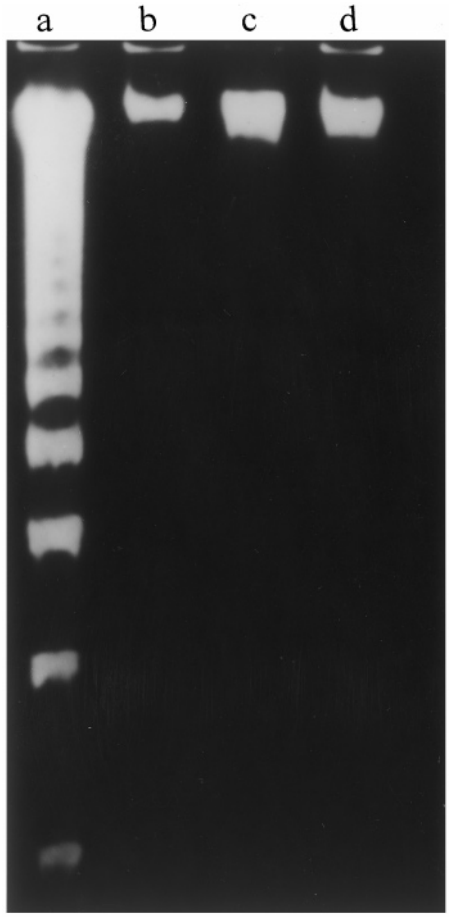

Figure 4. DNA fragments detected by agarose gel electrophoresis. *a.* DEX, 1×10^{-6} M; *b.* DEX (1×10^{-6} M) + DHEAS (1×10^{-4} M); c. control; d. DHEAS, $1 - 10^{-4}$ M.

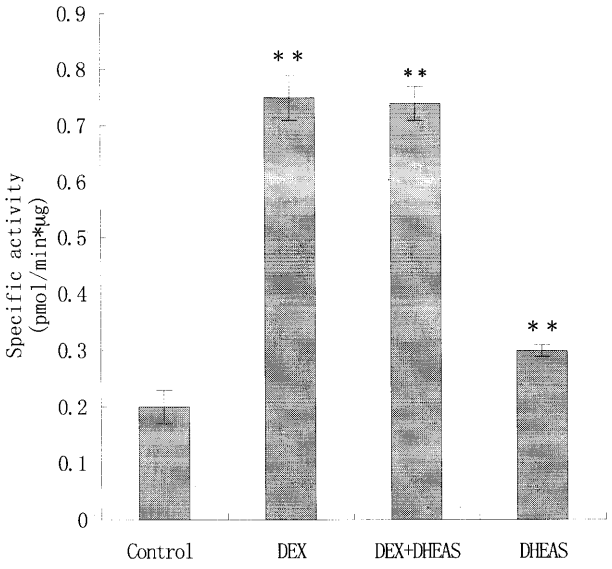

Figure 5. Specific activity of caspase-3. Values are the means of three separate experiments. Error bars indicate standard deviations. The level of significance was (**) $P < 0.01$ (Fisher's protected least-significant-difference, PLC). DEX, 1×10^{-6} M, DHEAS, 1×10^{-4} M.

Discussion

Although the global biological effects of DHEA have attracted a lot of attention, the cellular and molecular basis of its action remain elusive. Among these diverse effects, the protective effect of DHEA on thymus involution which accompanies the reduction of cell-mediated immune response may play one of the key roles in its action, forming the basis of many other biological effects such as protection against medical illness, infection and aging. There is little information with incomplete data regarding the inhibitory effect of DHEA on apoptosis in thymocytes. However, the cytological and biochemical evidence for this effect is far from being sufficient. In this paper we provide comprehensive and convincing data that DHEA strongly blocks thymocyte apoptosis induced by DEX.

It was reported that DHEA antagonizes glucocorticoid effects only under in vivo conditions [7], and the reasons for that is unclear. In our study, we found that the antiglucocorticoid effect of DHEA also occurred under in vitro conditions if the concentration used was not lower than 1×10^{-4} M. As mentioned by Kalimi et al., most of the DHEA effects reported have been obtained with pharmacological doses [7], which may account for the minimal information available regarding the mecha-

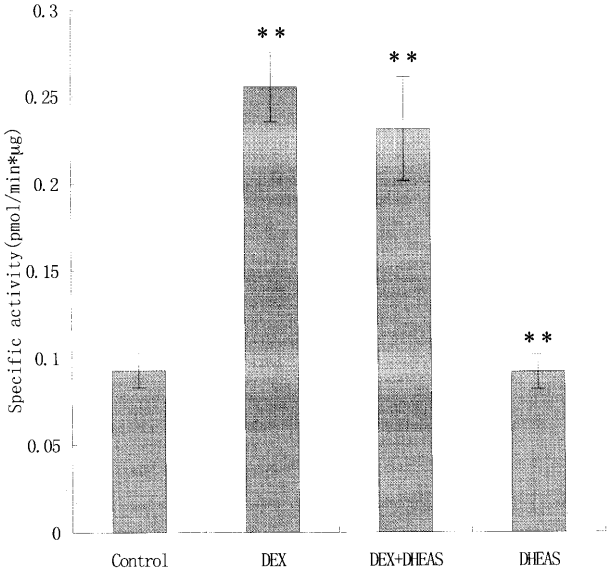

Figure 6. Specific activity of caspase-6. Values are the means of three separate experiments. Error bars indicate standard deviations. The level of significance was (**) $P < 0.01$ (Fisher's protected least-significant-difference, PLC). DEX, 1×10^{-6} M, DHEAS, 1×10^{-4} M.

nism of DHEA action. The use of a concentration as high as 100 μM may thus provide an explanation for the in vitro antiglucocorticoid effect of DHEA shown in this paper. Establishing the in vitro effect of DHEA will also facilitate the study of the cellular and molecular mechanisms of DHEA action. In addition, the effective concentration of DHEA is much higher than that for most hormones, including estrogen. This may exclude the possibility that DHEA functions as a precursor of sex hormones.

As reported in previous studies, pretreatment but not simultaneous treatment of DHEA is required to obtain antiglucocorticoid effects [6, 12]. The implication of this phenomenon is not yet clear. Our own results demonstrate that in the presence of DHEAS, activation of caspase-3 or caspase-6 by DEX is not disturbed at all, whereas DNA fragmentation is markedly blocked. On the other hand, since all the methods used for apoptosis detection are based on DNA cleavage, it is possible that modulation of apoptosis by DHEA may occur via the downstream events that finally lead to apoptotic DNA fragmentation.

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