Dehydroepiandrosterone Inhibits Complex I of the Mitochondrial Respiratory Chain and is Neurotoxic In Vitro and In Vivo at High Concentrations

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Dehydroepiandrosterone (DHEA) is widely used as a food supplement and considered to be relatively safe. In animal studies, however, additions of high concentrations of DHEA to the diet have led to hepatotoxicity as well as liver mitochondrial dysfunction. This study was therefore designed to find out whether DHEA is able to inhibit the respiratory activity also in neuronal mitochondria and to reveal whether this leads to functional disturbance in the brain. Using different mitochondrial substrates, we show here that DHEA suppresses the mitochondrial respiration in permeabilized neurons (half maximal inhibitory concentration \(13\mu M\)) by inhibiting complex I of the mitochondrial electron transport chain. Treatment with DHEA was associated with increased glucose expenditure in intact cultures and led to neuronal death. The latter was most prominent in hypoglycemic conditions. Mice fed with pellet containing 6% DHEA for 3 months showed a significant neuronal loss in the cerebral cortex and hippocampus, a slightly decreased dopamine/dihydroxyphenylacetic acid ratio, as well as motor impairment. The main conclusion of the present study is that high concentrations of DHEA inhibit complex I of the mitochondrial respiratory chain and are neurotoxic in vitro and in vivo.

Key Words: mitochondria; neurosteroids; neurodegeneration; neurotoxicity; complex I of the mitochondrial electron transport chain; dehydroepiandrosterone.

The level of dehydroepiandrosterone (DHEA), a natural precursor of male and female sex hormones in the human body, decreases dramatically with age. Abnormally low levels of DHEA have been reported to be related to a number of age-related diseases, and supplementary DHEA has been reported to have a variety of beneficial effects. These findings have led to DHEA’s popularity in the United States, where it is available as an over-the-counter dietary supplement, as well as in Europe. Although its recommended daily dosage ranges from 10 to 50 mg a day, DHEA is sold also in 100, 200, and even 400 mg tablets, and dosages up to 1600 mg are used. The highest reported daily dosages reach 6–8 g (Calabrese et al., 1990). Although DHEA appears to be relatively safe—administration of 1600 mg/day for 1 month to healthy volunteers caused only mild abnormalities in glucose metabolism but no other significant side effects (Mortola and Yen, 1990)—its long-term effects are unknown. In animal studies, however, addition of 0.6% DHEA to the diet of rats for 16 months (the equivalent human dose would be approximately 2000 mg/day) led to hepatotoxicity, which was associated with marked proliferation of mitochondria (Mayer and Forstner, 2004; Metzger et al., 1995). Earlier studies show that DHEA also inhibits respiration of isolated mitochondria supported by nicotinamide adenine dinucleotide (NAD)–dependent substrates (McIntosh et al., 1993; Mohan and Cleary, 1989; Sonka et al., 1978). Yang et al. (2002) have suggested that the antiproliferating effect of DHEA could be due to depletion of adenosine triphosphate (ATP). Altogether these results suggest that DHEA could impair the mitochondrial energy production. The present study was therefore undertaken to investigate whether DHEA could also impair neuronal energy metabolism, and if so, then to find out whether this could lead to neurodegenerative changes.

MATERIALS AND METHODS

Primary neuronal cultures. For the preparation of primary cultures of cerebellar granule cells, the cerebelli from 8-day-old Wistar rat pups were dissociated by mild trypsinization (0.25% trypsin at 35°C for 15 min) followed by trituration in a 0.004% DNase solution containing 0.05% soybean trypsin inhibitor. The cells were resuspended in Basal Medium Eagle with Earle’s Salts containing 10% heat-inactivated fetal bovine serum (FBS), 25 mM KCl, 2 mM glutamine, and 100 μg/ml gentamycin. The cell suspension was plated at a density of \(1.3 \times 10^5\) cells/ml on poly-l-lysine coated 35-mm dishes (2 ml/dish). Ten micromolar cytotoxic arabinoside was added 24 h after plating to prevent the proliferation of glial cells and the cells were cultured in a humidified 5% CO2/95% air at 37°C.

Primary cultures of rat mesencephalic cells were prepared from mesencephali of embryonic day 15 (E15) rats. Mesencephali were dissected and mechanically triturated with Pasteur pipette in Dulbecco modified Eagle...
medium (DMEM)/F12 medium containing 5% FBS and 100 µg/ml gentamycin, once centrifuged for 5 min at 4°C at 700 × g, resuspended in the same medium, and plated into 24-multiwell plates precoated with poly-L-lysine as 50 µl droplets with a density of 10^5 cells/ml. After 1 h, 0.5 ml of DMEM/F12 supplemented with 5% FBS was added. Twenty-four hours later the medium was changed to Neurobasal B medium with B27 supplement, containing 0.5mM glutamine and 100 µg/ml gentamycin, and half of the medium was changed every second day.

Primary cultures of rat cortical cells were prepared from fetal rat brain at E17. The cortices were dissected, triturated through a Pasteur pipette in DMEM/F12 supplemented with 5% FBS. This was followed by incubation for 5 min at room temperature (RT) until the formation of precipitates of undissociated cells, and supernatant containing dissociated cells was centrifuged for 5 min at 700 × g. The cells were resuspended in Neurobasal B medium supplemented with B27, glutamine, and gentamycin and plated into 2-ml culture dishes precoated with poly-L-lysine at a density of 10^6 cells/ml. The culture medium was changed every fourth day in vitro.

**Mitochondrial respiration.** Cultures of cerebellar granule cells were permeabilized by incubating cells with permeabilization medium (containing 7.23mM dipotassium salt of ethylene glycolbis-(beta-aminoethyl ether) N,N,N',N''-tetraacetic acid (K2EGTA), 2.77mM Ca2+EGTA, 6.56mM MgCl2, 0.5mM dithiothreitol, 50mM potassium 2-(N-morpholino)ethanesulfonate (K-MES), 20mM imidazole, 20mM taurine, 5.3mM Na2ATP, 15mM phosphocreatine, pH 7.1, and 50 µg/ml saponin) for 15 min at 4°C. The permeabilized neurons were then scraped from the plate, centrifuged at 4°C for 10 min at 1500 × g, the supernatant was removed, and the pellet was resuspended in permeabilization medium without saponin, centrifuged, and washed once more. Then the cells were transferred into the oxygraph medium (containing 7.23mM K2EGTA, 2.77mM Ca2+EGTA, 100mM MES, 20mM imidazole, 20mM taurine, 3mM K2HPO4, 0.5mM dithiothreitol, pH 7.1), with a final concentration of 5.66 million cells/ml and a total volume of 1.5 ml at 25°C. The oxygen consumption was recorded using an Oroboros oxygraph with Clark oxygen electrodes and analyzed using DATGRAPH software (Oroboros Instruments, Innsbruck, Austria). Respiration was initiated with the addition of different substrates (5mM glutamate and 2mM malate, or 5mM pyruvate and 0.5mM glutamine and 100 µg/ml saponin) to the DHEA-treated group was switched back to a standard diet without DHEA and the average dose of DHEA was 14 mg/per mouse/day. After 3 months, the diet of DHEA-treated group was switched back to a standard diet without DHEA for 1 month to exclude reversive effects of DHEA.

**Locomotor activity.** Locomotor activity was recorded in transparent cages (floor area 750 cm²) with a video tracking system (VideoMot 2; TSE, Germany) during 30 min and the total distance, locomotion time, resting time, and speed were calculated.

**Rotarod.** Motor coordination was measured using a rotarod. All mice were pretrained on the rotarod in order for them to reach a stable performance. The training consisted of four training sessions with a constant speed of 19 rpm over a 5-min period of two sessions on 2 consecutive days. The final test (10 sessions, each lasting 150 s, two sessions per each rpm) was performed on the third day at 14, 19, 23, 27, and 33 rpm. Between the trials, mice were given at least 10 min of rest in order to recover from stress and fatigue.

**Footprint.** The footprint pattern was used to compare the gait of control and DHEA-fed animals. To obtain footprints, the hind- and forefoot of the mice were coated with blue and green nontoxic paints, respectively. The animals were then allowed to walk along a 50-cm-long and 6-cm-wide runway (with 10-cm-high walls) into an enclosed box. A fresh sheet of white paper was placed on the floor of the runway for each run. Stride length was measured as the average distance of forward movement between each stride.

**Assays for neuronal death.** The neuronal death was quantitatively assessed using the Trypan Blue exclusion method. The cultures were washed with PBS (145mM NaCl, 3mM KCl, 0.42mM Na2HPO4, 2.4mM KH2PO4, pH 7.4), incubated with a 0.4% Trypan Blue solution at RT for 10 min and washed twice with PBS. Stained culture dishes were mounted on the motorized microscope stage of an inverted microscope (BSX1 with ×20 lenses; Olympus, Japan) connected with a video camera, and five to eight random fields (305 × 323 µm), each containing 100–200 neurons, were photographed. The images were later analyzed by an independent observer using the Computer Assisted Stereological Toolbox (CAST) program (Olympus, Denmark), and the number of Trypan Blue–positive cells and total number of cells were counted.

To stain the dopaminergic neurons, the mesencephalic cultures were subjected to tyrosine hydroxylase immunohistochemistry as described below and the total number of tyrosine hydroxylase positive neurons in one droplet (approximately 400 tyrosine hydroxylase positive cells per droplet) was counted. At least four dishes were used for each data point and experiments were performed at least twice.

To detect the occurrence of the neuronal death in vivo, 40-µm brain sections were immersed in 100% ethanol for 3 min and dehydrated with graduated alcohol solutions. The sections were then incubated in a solution of 0.001% Fluoro-Jade (Histo-Chem Inc, AR) in 0.1% acetic acid for 30 min. Sections were then rinsed in water, air dried, cleared in xylene, coverslipped, and examined with an epifluorescence microscope with a filter designed to detect fluorescein.

**Animals and DHEA treatment.** Male Balb/c mice, 12 weeks old at the start of the experiment, were housed individually in plastic cages at 22°C in an animal room with a 12-h light-dark cycle (lights on at 9:00 A.M.). The mice were randomly assigned to either the control group or the DHEA-treated group. The control group was fed with a standard diet, a rodent diet (AIN-76), while the DHEA-treated group was given an AIN-76 diet containing 0.6% of DHEA (Research Diets, Inc., NJ). The animals were given ad libitum access to food and water. The remaining pellet as well as animals were weighed once a week and the average dose of DHEA was 14 mg/per mouse/day. After 3 months, the diet of DHEA-treated group was switched back to a standard diet without DHEA for 1 month to exclude reversive effects of DHEA.

**Complex 1 activity.** To measure the mitochondrial NADH ubiquinone oxidoreductase activity, the cultured cerebellar granule cells were gently washed with prewarmed 0.1M phosphate-buffered saline (PBS), scraped from the dishes (about 10^7 cells), and centrifuged for 3 min at 1500 × g at 4°C. Pellet was resuspended in 1 ml of ice-cold 250mM sucrose buffer (containing 2mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and 0.1mM EGTA, pH 7.4), homogenized with Teflon homogenizer, and then centrifuged for 6 min at 1500 × g. The pellet was rehomogenized, centrifuged, and supernatant from both centrifugation was collected and centrifuged at 14,000 × g for 10 min at 4°C. Pellet was resuspended in 100 µl of 25mM potassium phosphate buffer (pH 7.4) and frozen and thawed twice before analysis. Five microliters of suspension was used for enzyme activity measurement in 300 µl of mixture containing 25mM potassium phosphate buffer (pH 7.4), 0.25% bovine serum albumin, 2mM KCN, and 60µM decylubiquinone. The reaction was started by an addition of 0.2mM NADH and the NADH oxidation was measured at 37°C at a 340 nm wavelength for 5 min with 30-s intervals using a computer-controlled spectrophotometer Spectronic GENESYS 5 (Thermo Electron Corporation, MA). The drugs were added simultaneously with the NADH. The rotenone-sensitive part (5pM) of the NADH dehydrogenase activity was taken to represent the activity of complex I.

**Glucose and lactate concentrations.** Glucose was measured spectrophotometrically by enzymatic assay using hexokinase coupled with glucose-6-phosphate dehydrogenase (Kunst et al., 1988), and l-lactate was measured using lactate dehydrogenase coupled with alanine transaminase as described by Noll (1988).
Cell counting. Right hemispheres were fixed in 4% paraformaldehyde for at least 48 h and sectioned by a vibratome (Leica VT1000, Germany) in the coronal plane at 40 μm. Each sixth section was stained by eosin-hematoxylin and the neuronal density was estimated using CAST Grid 2.0 software. Nuclei were counted in optical dissectors (20 × 20 μm², with a 20-μm depth [25 μm for CA1]) using systematic random sampling (distance between the frames was 200 μm in motor cortex, 30 μm in CA1, and 500 μm in striatum) (West et al., 1991). The regional boundaries of the structures were delineated according to the mouse brain atlas. The observed coefficient of error varied from 0.044 to 0.078.

For tyrosine hydroxylase staining, the sections were incubated with 0.6% H₂O₂ in PBS for 30 min, followed by blocking in 2% goat serum in PBS containing 0.5% Tween-20 and 0.25% Triton-X-100 (Blocking buffer). Then, sections were incubated for 48 h at 4°C with rabbit polyclonal antityrosine hydroxylase (1:1000, Chemicon International, UK) in blocking buffer. This was followed by incubation with biotinylated goat anti-rabbit antibody (1:200) in blocking buffer for 1 h at 37°C and then in avidin-biotin-peroxidase complex (Vector Laboratories, UK) in 10mM phosphate buffer for 30 min at RT. Tyrosine hydroxylase–positive neurons were visualized using 3,3-diaminobenzidine (Vector Laboratories). The number of tyrosine hydroxylase positive cells was counted in a sampling volume 60 × 60 × 15 μm and the numerical density (N/mm³) was calculated. Anatomical boundaries of substantia nigra were delineated as follows: the medial border was defined by a line passing through the medial tip of the cerebral peduncle; the ventral border followed the dorsal boundary of cerebral peduncle; the dorsal border passed above pars compacta and below the ventral margin of the medial lemniscus.
partly the mitochondrial respiration supported by hydroxybutyrate, a substrate for both complex I and II in the brain. These observations strongly suggest that DHEA suppresses the mitochondrial respiration by inhibiting complex I.

To further test this assumption, we isolated functional complex I (NADH quinone oxidoreductase, Enzyme Commission Number 1.6.99.3) from the remaining part of the respiratory chain using decylubiquinone (oxidized quinone) as the complex I electron acceptor, in the presence of KCN, and measured the effects of DHEA on its activity. As shown in Figure 1G, electron transfer through complex I was inhibited by DHEA. The IC50 value for DHEA (35 ± 2 μM) was considerably lower than for complex I inhibitor 1-methyl-4-phenylpyridinium (MPP+) (2.2 ± 0.5mM), known to induce Parkinson’s disease in humans and used by us as a positive control.

The observed effect of DHEA on complex I activity and mitochondrial respiration was specific not only for this compound but also for some other neurosteroids. Progesterone inhibited complex I at similar concentrations as DHEA (IC50 39 ± 3 μM), pregnenolone (IC50 90 ± 24 μM), and estradiol (IC50 116 ± 3 μM) were weaker, and DHEAS and cholesterol (IC50 > 350 μM) were ineffective. Also, the DHEA metabolites 7-alpha-hydroxy-DHEA, 7-beta-hydroxy-DHEA, and 7-keto-DHEA, were ineffective (IC50 between 290 and 360 μM).

**DHEA Affects the Energy Metabolism in Intact Neurons**

Since inhibition of mitochondrial function leads to an activation of glycolysis to compensate for the loss of mitochondrial ATP supply (Pauwels et al., 1985), we tested whether DHEA also is able to alter the balance between oxidative phosphorylation and glycolysis by measuring its effects on glucose consumption and lactate accumulation in intact neurons. Figure 2 demonstrates that treatment of cerebellar granule cells with 100 μM DHEA led to accelerated depletion of glucose from the medium when compared with the untreated neurons, which shows activation of glycolysis. Parallel measurements demonstrating lactate accumulation suggest that after treatment with DHEA the efficiency of mitochondria to use pyruvate was decreased, as more than 40% of pyruvate was converted to lactate. Thus, in the presence of DHEA the energy metabolism was shifted from oxidative phosphorylation to glycolysis, which was less efficient in ATP production, and which could impair the neuronal survival under metabolically stressful conditions.

**DHEA Induces Neuronal Death In Vitro**

Our next task was to reveal whether the observed disturbances in neuronal energy metabolism could lead to death of the neurons. Figure 3 demonstrates that 24 h incubation with 0–100 μM DHEA in the presence of high glucose concentration (>1 mM) did not compromise the viability of cortical and cerebellar neurons. In culture of mesencephalic neurons, however, 24 h incubation with 100 μM DHEA caused a death in more than half of the tyrosine hydroxylase positive cells. Figure 3D demonstrates that in cerebellar neurons initially nontoxic 100 μM concentration of DHEA became toxic after 72 h incubation suggesting that toxicity increases when increasing the exposition time. Moreover, under hypoglycemic conditions (<1 mM) DHEA compromised the neuronal survival even at as low as 3.3 μM concentration (Fig. 3E). The latter effect was reversed fully when glucose (2.8 mM) or partially when the flavine adenine dinucleotide-linked substrate, succinate (5 mM), was added to the medium (data not shown).

**DHEA Induces Neuronal Death In Vivo**

Male mice were fed with a diet containing 0.6% DHEA for 10 weeks (average daily dosage 14 mg) and then 4 weeks with a normal diet to exclude acute effects of DHEA. No Flouro-Jade signal specific to degenerating cells was observed in brains of control and DHEA-fed animals after that period. However, as earlier neurodegeneration could have been undetected while visualizing degenerating neurons only, we also estimated the neuronal density in hippocampal CA1 region, primary motor cortex, and in striatum, i.e., in the regions, which are shown to be sensitive to energetic stress. As Figure 4 demonstrates, neuronal density was significantly lower in primary motor cortex (221,600 ± 7400 cells/mm³ in DHEA vs. 267,900 ± 5000 cells/mm³ in controls, p < 0.001, n = 8) and in hippocampal CA1 region (576,600 ± 10,200 in DHEA vs. 613,700 ± 12,800, p < 0.001, n = 8).
but not in striatum (210,000 ± 10,000 in DHEA vs. 216,500 ± 11,600 in controls, \( p = 0.68, n = 8 \)).

**DHEA Induces Motor Impairment**

The motor function was evaluated by measuring locomotor activity, rotarod performance, and gait in DHEA-fed mice after 4 weeks of DHEA discontinuation. During a 30-min locomotor activity test, the DHEA-treated mice displayed decreased total distance traveled (5.46 ± 0.23 vs. 6.30 ± 0.30 m in controls, \( p = 0.046, n = 9 \)), decreased movement speed (5.00 ± 0.11 cm/s vs. 5.61 ± 0.19 cm/s in controls, \( p = 0.016, n = 9 \)), but no change in the movement time (1120 ± 30 s vs. 1090 ± 40 s in controls, \( p = 0.53, n = 9 \)). The motor coordination and balance of mice were measured using a Rotarod test. Both DHEA-fed and control mice performed well in the Rotarod test at rotation speeds up to 20 rpm, remaining on the rod almost for a full 150-s trial. However, at higher rotation speeds the DHEA-fed mice fell down more frequently than control mice (Fig. 5). Figure 5B shows that the overall performance of DHEA-fed mice in Rotarod test, calculated by summarizing the performances at different Rotarod speeds, was 25% less than in the control mice (\( p = 0.01, n = 9 \) in both groups). Gait disturbance was assessed by analyzing the footprint patterns while the mice walked along a narrow corridor. The control mice walked in a straight line with a regular even alternating gait, placing the hindpaw close to the position where the ipsilateral forepaw had been in the previous step (Fig. 5C). By contrast, DHEA-fed mice walked...
with blue (black) and green (gray) nontoxic paints, respectively.

**FIG. 5.** DHEA impairs motor function. (A) Average time of mice spent in rotarod at different rotation speeds (control—filled circles, DHEA-fed—open circles). (B) Scatter blot of overall performance of control and DHEA-fed mice in rotarod. *p < 0.05 compared with untreated mice. (C) Footprint patterns of control and DHEA-fed animals. Hind- and forefeet of the mice were coated with blue (black) and green (gray) nontoxic paints, respectively.

DISCUSSION

Our study demonstrates that DHEA inhibits mitochondrial respiration by directly acting on complex I of the respiratory chain. These results support earlier observations (McIntosh et al., 1993; Mohan and Cleary, 1989; Sonka et al., 1978) demonstrating inhibitory effect of DHEA on the respiration of isolated liver mitochondria. However, the present report is the first to show that complex I represents a target for DHEA in association with its strong neurotoxic effects.

The IC_{50} of DHEA observed in inhibition of complex I (35μM) is by orders of magnitude higher than the IC_{50} for rotenone (5–30nM) and acetogenins (1–10nM), the specific complex I inhibitors (Champy et al., 2004; Tormo et al., 2003) but considerably lower than for MPP{\textsuperscript{+}} (0.3–4mM; Nicklas et al., 1985; Ramsay et al., 1989) or metformin (80mM; Owen et al., 2000). Rotenone, acetogenins, and 1-methyl 4-phenyl 1,2,3,6-tetrahydropyridine (MPTP, “prodrug” of MPP{\textsuperscript{+}}) are relatively lipophilic molecules which pass the blood-brain barrier and inhibit mitochondrial respiration of the complex I of the respiratory chain in brain mitochondria. MPP{\textsuperscript{+}} is known to induce Parkinson-like symptoms in humans and acetogenins have been linked to Guadeloupean parkinsonism associated with frontolimbic dementia and amyotropic lateral sclerosis (Caparros-Lefebvre et al., 2002; Champy et al., 2004; Lannuzel et al., 2003). It has been demonstrated that chronic systemic exposure to rotenone induces features of Parkinson’s disease in the rodents. Previous epidemiological studies have also suggested an association between the pesticide use and the prevalence of Parkinson’s disease (Gorell et al., 1998). Regarding metformin, it is known that it is a much weaker complex I inhibitor and less lipophilic than those mentioned above, but its penetration of the blood-brain barrier has not been studied. However, elevated plasma lactate in metformin-treated patients (Davis et al., 2001; Musi et al., 2002), in some instances aggravating to lactic acidosis (Luft, 2001), suggests that metformin inhibits complex I in the peripheral tissues.

Our findings show that the neurotoxicity pattern produced by DHEA is similar to that for other complex I inhibitors. Like the latter compounds, DHEA-impaired energy metabolism in cultured neurons and induced neuronal death in different types of neuronal cultures, this effect being most evident in hypoglycemic conditions. An addition of 0.6% DHEA in the diet of mice for 3 months led to a neuronal loss and induced behavioral deficits. Impaired performance in Rotarod and stride length tests, and decreased locomotor activity in open field test together with a decreased DA/DOPAC ratio suggest the damage to the nigrostriatal dopaminergic system. At the same time a 20% decline in DA/DOPAC ratio and a 25% decline in Rotarod performance were not as prominent as usually observed in MPTP or rotenone-treated mice.

The inhibitory action of DHEA on mitochondrial complex I that we observed could also cast some light on the mechanisms of other known effects of DHEA. Administration of DHEA to rodent models has many beneficial effects, including antiaging, antidiabetic, anticancer, and antiatherogenic properties when...
used at pharmacological doses. In addition, accumulating evidence suggests that DHEA(S) is neuroprotective in a variety of paradigms (Baulieu and Robel, 1998; Baulieu et al., 2000). It was recently shown that DHEA(S) is neuroprotective in *in vitro* and *in vivo* (Kaasik et al., 2001; Kimonides et al., 1998; Li et al., 2001) models of brain ischemia. Nevertheless, the mechanisms through which DHEA(S) exerts these effects have still not been unraveled. No specific receptors in the plasma membrane, nucleus or cytoplasm for DHEA(S) have been found. It has been shown that DHEA and its sulfate modulate GABAergic and glutamatergic neurotransmission. However, while the neuroprotective effect of DHEAS might be attributed to its γ-aminobutyric acid (GABA_α) receptor–modulating properties (Lapchak et al., 2000), DHEA itself is not interacting with the GABA_α receptor (Sousa and Ticku, 1997). The neuroprotective effects of DHEA seem not to be related with its N-methyl-D-aspartate or sigma receptor–activating properties as well (Bergeron et al., 1996; Debonnel et al., 1996). Thus, the neuroprotective effects of the DHEA cannot be explained at the receptor level of the above-mentioned signaling systems. Instead, our data allow to explain several of the DHEA effects by its inhibitory action of DHEA on mitochondrial complex I: (1) it has been shown that dietary rotenone reduces the background incidence of liver tumors in mice (Cunningham et al., 1995) and mammary tumors in rats (Hansen et al., 1965) and prevents cell proliferation induced by a peroxisome proliferator in the livers of mice (Cunningham et al., 1995). Several acetogenins inhibit proliferation of human tumor cell lines by inducing apoptosis (Chih et al., 2001; Chiu et al., 2003; Zhu et al., 2002) and recently it was shown also that metformin reduces the risk of cancer in diabetic patients (Evans et al., 2005). It could be thus speculated that DHEA, like other complex I inhibitors, might have antiproliferative and anti-tumorigenic effects. This hypothesis is supported by experimental data showing that DHEA inhibits, at pharmacological concentrations, the proliferation of B16 mouse melanoma cells (Kawai et al., 1995), human breast cancer cells (Di Monaco et al., 1997; Gayosso et al., 2006), and neuroblastoma cells (Silvagno et al., 2002). DHEA reduces also the number and proliferation of preneoplastic and neoplastic lesions in rat liver induced by N-nitrosomorpholine (Mayer et al., 2003; Weber et al., 1988). It has been already suggested that some of these antiproliferative effects might be due to the DHEA-induced depletion of ATP (Yang et al., 2002). This hypothesis is, however, opposed by observations that DHEA is hepatocarcinogenic (Metzger et al., 1995) in rats. Recent result by Kopplow et al. (2005) demonstrates also that, in short-term treatments, DHEA is a liver mitogen in rats although it has antiproliferative effect in long-term treatments. (2) The inhibitory effect of DHEA on complex I may also partly explain possible antidiabetic effects of DHEA. Metformin, as well as thiazolidinediones, used for the treatment of type 2 diabetes, inhibit mitochondrial complex I (Brunmair et al., 2004; Owen et al., 2000) and it has been suggested that this could contribute to the antidiabetic actions of these drugs. Similarly to DHEA, thiazolidinediones also reduce aerobic fuel consumption and accelerate anaerobic glycolysis (Brunmair et al., 2001; Coates et al., 2002; Dello Russo et al., 2003; Fürniss et al., 2000; Preiminger et al., 1999). (3) Oddly enough, the antisemich effect of DHEA could be associated with its inhibitory action on mitochondrial complex I. A reversible blockade of the electron transport chain at complex I by amobarbital during ischemia protects mitochondrial respiration (Chen et al., 2006). Also an irreversible blockade by rotenone decreases ischemic damage to mitochondria by decreasing the loss of cytochrome c and preserving respiration through cytochrome oxidase (Ichikawa et al., 2004; Lesniewsky et al., 2004). Observed protective effect of DHEA in different models of brain ischemia (Aragno et al., 2000; Kaasik et al., 2001; Li et al., 2001) could be thus partially explained by a reversible blockade of complex I. Our earlier observations that DHEA inhibits Ca^{2+} influx into the mitochondrial matrix (Kaasik et al., 2003) could be also explained by a blockade of the electron transport chain.

The positive effects of DHEA described above could justify its use in treatment of human diseases. Indeed, in the United States, and now increasingly in Europe, DHEA is one of the most commonly used dietary supplements. The most commonly recommended daily dosage for DHEA is between 10 and 50 mg and it seems unlikely that DHEA could exert the above-mentioned toxic effects at near physiological concentrations. However, DHEA is used frequently in much higher dosages (up to 1600 mg/day), which result in highly supra-physiological concentrations. At the same time, its potential toxic relevance in humans is not yet defined. Our data suggest that prolonged exposure to high dosages of DHEA may bring about a too-strong inhibition of the complex I of the mitochondrial respiratory chain in the human brain, and thereby cause neurotoxic alterations. Such an adverse effect of DHEA can be increased due to its lipophilic nature that eases its passing through the blood-brain barrier.

The main conclusion of the present study is that DHEA inhibits complex I of the mitochondrial respiratory chain and is neurotoxic *in vitro* and *in vivo* at high concentrations. These results stress the need for epidemiological studies to identify whether the long-term use of high doses of DHEA is associated with neurological complications.

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