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Dehydroepiandrosterone effects on Akt signaling modulation in central nervous system of young and aged healthy rats

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ABSTRACT

Dehydroepiandrosterone (DHEA) is a steroid synthesized in adrenal cortex as well as in the nervous system. DHEA effects on central nervous system (CNS) have been associated with several brain functions such as marked neurotrophic and neuroprotective activity. DHEA plasma concentration decreases steadily with aging and studies have reported an inverse correlation between levels of DHEA and neurological diseases age-associated. Nonetheless, its mechanisms of action are not yet fully understood. Akt signaling pathway is one protein kinase which has been related to be DHEA modulated. The goal of this study was to investigate whether short-term (6 or 24 h) or chronic (5 weeks) DHEA treatment modulates Akt in CNS of adult (3 months) and aged (18 and 24 months) healthy rats. Hypothalamus and hippocampus homogenates were prepared to quantify total-Akt and phosphorylated Akt at Ser^{473} (pAkt). The results here presented have shown that acute (50 mg/kg) and chronic (10 mg/kg) DHEA injections modulate total and pAkt levels. This effect was dose and time-dependent as well as age and tissue-dependent. In addition, the age variable also intervenes on total and pAkt levels expression independently of DHEA treatment.

1. Introduction

Aging is characterized by the declining ability of organ systems to respond to stress, increasing homeostatic imbalance and pathology incidence. Changes in the aging brain mainly affect cell functions, neuritic density and synapse numbers [1]. In aging individuals, the endocrine changes result in decrease of endocrine function involving the tissues responsiveness as well as reduced hormone secretion from peripheral glands [2]. The secretion and the blood levels of the adrenal steroid dehydroepiandrosterone (DHEA) and its sulfate ester (DHEAS) decrease with age [3]. In addition to adrenal synthesis, DHEA and DHEAS are synthesized in the central nervous system (CNS), suggesting a role of these hormones in brain function and development [4,5]. Changes of DHEA and DHEAS levels could have a negative impact on brain function. Indeed, decreased DHEA and DHEAS contents have been reported in Alzheimer's disease and dementia in human brain [6]. It has been proposed that DHEA replacement may have anti-ageing effects and improve wellbeing and sexual function [7,8].

Several lines of evidence suggest the relevance of DHEA and DHEAS for neuronal function due to the fact that these hormones could be involved in the processes of neuronal survival, exerting neuroprotective activity in a variety of both in vitro and in vivo experimental paradigms. In vitro, DHEA and DHEAS have been shown to attenuate excitotoxicity, protecting neuronal cells against excitatory amino acids and glucocorticoid toxicity [9–11]. Moreover, DHEA and DHEAS have been shown to be neuroprotective against damage induced by oxidative stress in human neuroblastoma cells [12]. In vivo, DHEA and DHEAS protect brain against ischemia [13]. In diabetic rats, chronic DHEA administration exerts antioxidant effects, reducing the brain damage and ameliorating several markers of neuronal damage induced by oxidative stress [14,15]. Although no specific receptors in the plasma membrane, nucleus or cytoplasm for DHEA have been clearly found, this steroid could act through several mechanisms to exert its functions [16]. In neurons, the prosurvival effect of DHEA(S) appears to be NMDA- [17]; GABAA- [11,18] and sigma1-dependent [10,19]. It has also been described a DHEA antiapoptotic effect mediated by G-protein-coupled-specific membrane binding sites in neurons [20,21].

Potential mechanisms for neuroprotective effects are suggested by the observation that DHEA and DHEAS modulate specific signal

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transduction pathways involved in cell survival. DHEA has been found to activate the phosphatidylinositol 3-kinase (PI3K) cascade and Akt, also called protein kinase B (PKB), a key downstream effector of PI3K in mediating cell survival [11,12,18,22].

Akt, a serine/threonine kinase, plays a pivotal role in transducing a variety of extracellular stimuli (growth factors and cytokines) into a wide range of cellular processes, including metabolism, cell proliferation and apoptosis [23]. The activation of PI3K stimulates Akt phosphorylation at two residues: Thr 308 in the kinase domain and Ser^{473} in the hydrophobic motif. Akt is widely implicated in neuronal survival through inhibition of apoptosis [11,22,24]. Akt activation could be changed in aging by alterations in signalizing molecules, as well to reduced cellular response due receptors down-regulation and also by functional decrease in hormonal systems [25–27]. Nie et al. [28] have suggested a potential link between the age-related alteration of Akt (Ser^{473}) and the deterioration in performance of learning and memory tasks in SAMP10 mouse. Furthermore, the neurotrophic and antiapoptotic Akt mediated effects could be a future therapeutic approach to neurodegenerative disorders treatment like Parkinson's and Alzheimer's diseases [28–30].

Besides the effects on the CNS, DHEA may be considered as a multifunctional steroid with protective effects in many aspects of cellular dysfunction, especially aging-associated deficits, such as diabetes, cancer, atherosclerosis and immunological decline and it could explain the aggressive marketing of DHEA as a source of youth [7]. Indeed, there is widespread commercial availability of DHEA outside the regular pharmaceutical networks in the United States as a dietary supplement without medical care [31]. Because there is a considerable increase in life expectancy, is important to development new therapeutical strategies to promote "successful aging" – defined as low probability of disease, high cognitive and active engagement in life. DHEA has been the focus of many studies, but DHEA replacement in the elderly and the mechanisms of action of DHEA at the molecular and cellular levels are still unclear [31]. In addition, its in vivo effects on intracellular cascades modulation on the CNS are not fully explained too. The main goal of the present work was to investigate whether in vivo DHEA administration – using different experimental schedules – may modulate Akt expression in hypothalamus and hippocampus of adult and aged rats.

2. Materials and methods

2.1. Animals

Wistar male rats (3, 18 and 24 months) were obtained from the Central Animal House of the Universidade Federal do Rio Grande do Sul. Animals were housed in plastic cages (4 animals each) and received water and pelleted food ad libitum. They were maintained under standard laboratory conditions (controlled temperature of 21 °C, 12 h light/dark cycle). Special care was taken to minimize the number of animals used and their suffering. All animal procedures used in this study were in accordance with the Principles of Laboratory Animal Care (COBEA—Brazilian College of Animal Experimentation) and were approved by the Ethics Committee at the Federal University of Rio Grande do Sul.

2.2. Drugs

Dehydroepiandrosterone (DHEA, CALBIOCHEM, San Diego, EUA) was dissolved in 20% of 2-hydroxypropyl-(-cyclodextrin (FLUKA, Sigma–Aldrich, SP) to acute treatment and in vegetal oil (Special Salad, Bünge) to chronic treatment. All solutions were prepared at the same day of injections.

2.3. Experimental protocol

This study was divided in two steps using different experimental protocols:

2.3.1. Acute dose–response curve

DHEA acute administration protocol was based on a recent study from our group [32] which states that there are selective changes between the two time points (6 and 24 h) of DHEA administration related to Akt activation. Adult rats (3 months) were injected intraperitoneally (i.p) with 1, 5, 10 or 50 mg/kg/dose of DHEA or with vehicle (2-hydroxypropyl-(-cyclodextrin). Six or twenty-four hours after the injections, animals were killed to remove the brain structures (hyphothalamus and hippocampus).

2.3.2. Chronic treatment

The purpose of this experimental protocol was to analyze the effect of chronic DHEA administration on Akt signaling in rats CNS with different ages. Three, eighteen and twenty-four months-old rats were injected subcutaneously with DHEA (10 mg/kg) or vehicle (vegetal oil), once a week, for 5 weeks. DHEA chronic administration protocol was based on other studies [33–35]. Each age group experiment was performed using 3 months untreated rats as a control group for comparison.

2.4. Tissue samples preparation

After each experimental protocol, rats were killed by decapitation and their hypothalamus and hippocampus were quickly dissected out. The tissues were immediately frozen on dry ice and stored at −70 °C for Western blot assay. Hypothalamus and hippocampus were homogeneized in lysis buffer (pH 7.4) containing protease inhibitors and detergents (20 mM Tris–HCl, 150 mM NaCl, 1 mM EDTA, 10% glycerol, 1% Nonidet P40, 200 μM phenylmethylsulfonil fluoride (PMSF), 25 μg/mL leupeptin). The homogenates were centrifuged at 3000 × *g* for 10 min at 4 °C (Sorval RC 5B-rotor, SS-34, Du Pont Instruments, EUA) to discard nuclei and cell debris, and the supernatant fraction obtained was used to Western blot assay.

2.5. Western blotting

After protein measurement [36] sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on 10% (w/v) was carried out using a miniprotein system (BIO-RAD, Hercules, CA, USA) with broad range molecular weight standards (Amersham). Protein (90 μg) was loaded in each lane with loading buffer containing 65 mM Tris (pH = 6.8), 50% glycerol, 10% SDS, 0.5 M mercaptoethanol, 0.002% bromophenol blue. Samples were heated at 90 °C for 2 min prior to gel loading. After electrophoresis, proteins were transferred to nitrocellulose membranes (Hybond, Amersham) using an electrophoretic transfer system (BIO-RAD). The membranes were then incubated with 1% BSA dissolved in TTBS (20 mM Tris–HCl, 140 mM NaCl, 0.05% Tween-20, pH 7.4) for 60 min. The membranes were incubated over night at 4 °C with the primary antibodies diluted in TTBS plus 2.5% BSA and 1% sodium azide. Goat anti-total-Akt polyclonal antibody (1:1000) and rabbit anti-phospho Akt (Ser^{473}) (1:500), Santa Cruz Biotechnology, were used as primary antibodies. After washing with TTBS, the membranes were incubated for 2 h at room temperature with secondary antibody (1:10,000, anti-goat and anti-rabbit IgG peroxidase conjugated; Santa Cruz Biotechnology and Zymed Laboratories) and washed with TBS (20 mM Tris–HCl, 140 mM NaCl, pH 7.4). The membranes were revealed by chemiluminescence followed by apposition of the membranes to autoradiographic films (Hyperfilm ECL, Amersham). These films were analyzed with an image

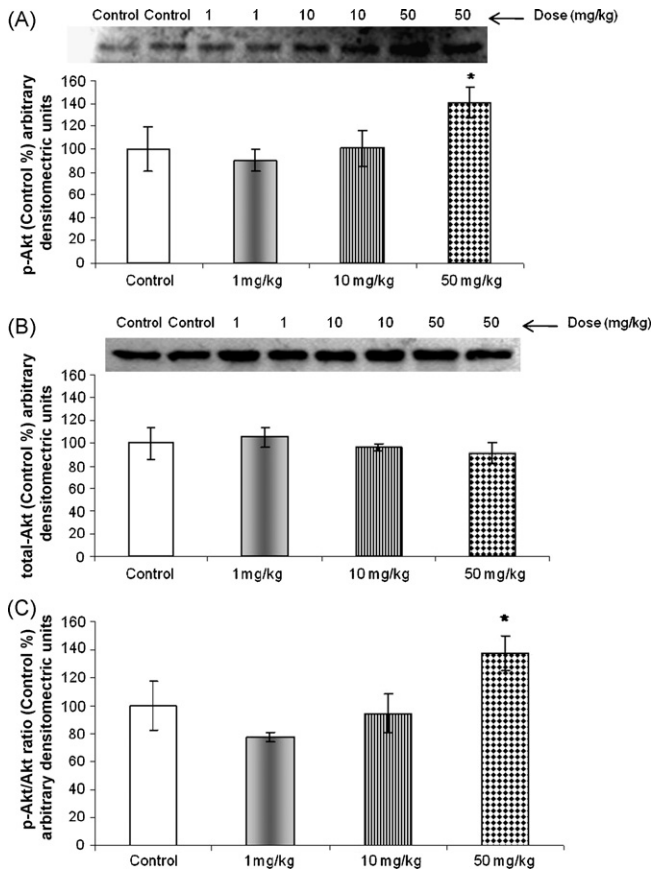

Fig. 1. Western blot analysis in hippocampus after 6 h of DHEA treatment. (A) pAkt; (B) total-Akt; (C) pAkt/total-Akt ratio. $n = 3$–4 for each group. *Significantly different from all tested groups ($P < 0.05$).

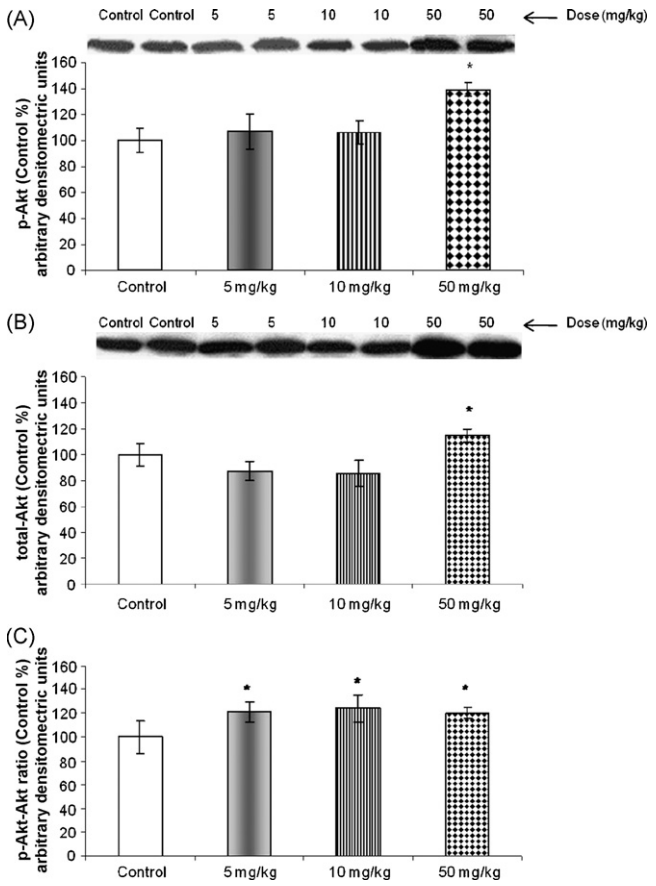

Fig. 2. Western blot analysis in hippocampus after 24 h of DHEA treatment. (A) pAkt; (B) total-Akt; (C) pAkt/total-Akt ratio. $n = 4$ for each group. *Significantly different from all tested groups ($P < 0.05$).

densitometer (Image Master VDS Software version, Amersham). The results from each membrane were normalized to the Ponceau values (5% in acetic acid) instead of using actin or tubulin as control, once DHEA could alter cytoskeleton proteins [37]. To minimize interassay variations, samples from all experimental groups were processed in parallel ($n = 3$–5 in each group).

2.6. Statistical analysis

All data are expressed as mean ± SD, considering 3 months-control group of each experiment 100%. Comparisons of results between treatments over different experimental schedules were done using either one- or two-way analyses of variance (ANOVA), as appropriate. The statistical significance was determined using the Student–Newman–Keuls post hoc multiple comparison test. Differences were considered statistically significant when P value <0.05. All statistical analyses were carried out with Sigma Stat 2.0 software.

3. Results

3.1. Hippocampus

3.1.1. Acute treatment

Figs. 1 (6 h) and 2 (24 h) present dose-dependent response after DHEA acute treatment on pAkt, total-Akt and pAkt/Akt ratio in hippocampus of adult rats (3 m). PAkt significantly increased after 6 and 24 h of DHEA treatment in the groups treated with the highest tested dose, 50 mg/kg, as compared to control, 1, 5 and 10 mg/kg groups (Figs. 1A and 2A). Total Akt level was also enhanced after 24 h of 50 mg/kg of DHEA treatment (Fig. 2B) but no change was significant on total-Akt level after 6 h (Fig. 1B). PAkt/Akt ratio was also increased after 24 h of 5, 10 and 50 mg/kg of DHEA treatment as compared to control group, but only 50 mg/kg of DHEA significantly increased pAkt/Akt ratio after 6 h (Figs. 1C and 2C).

3.1.2. Chronic treatment

Figs. 3 (3 and 18 month-old) and 4 (3 and 24 month-old) show data from Western blot analysis of total and pAkt measured in the hippocampus of rats with different ages after 5 weeks of DHEA (10 mg/kg) administration. There was no change of pAkt as well as in its total content in the hippocampus of 3 month-old treated group when compared to its control. It is noteworthy that this result was similar in all experiments (Figs. 3 and 4).

There was a reduction (around 13%) on pAkt in hippocampus of 18 month-old control group when compared to 3 month-old control group and DHEA treatment enhanced pAkt in 18 month-old group when compared to its control (Fig. 3A). There was no significant difference between the 18 month-old control and the 3 month-old control groups regarding total-Akt content in the hippocampus. Total-Akt content enhanced after DHEA treatment in hippocampus of 18 month-old treated rats (about 27%) if compared to 18 month-old control (Fig. 3B). There was a reduction on pAkt/Akt ratio in hippocampus of 18 month-old control group as compared to 3 month-old control group (Fig. 3C).

There was no change of pAkt in hippocampus of 24 month-old control group when compared to 3 month-old control group and it was significantly increased in 24 month-old DHEA group when compared to 24 month-old control group (Fig. 4A). Total Akt

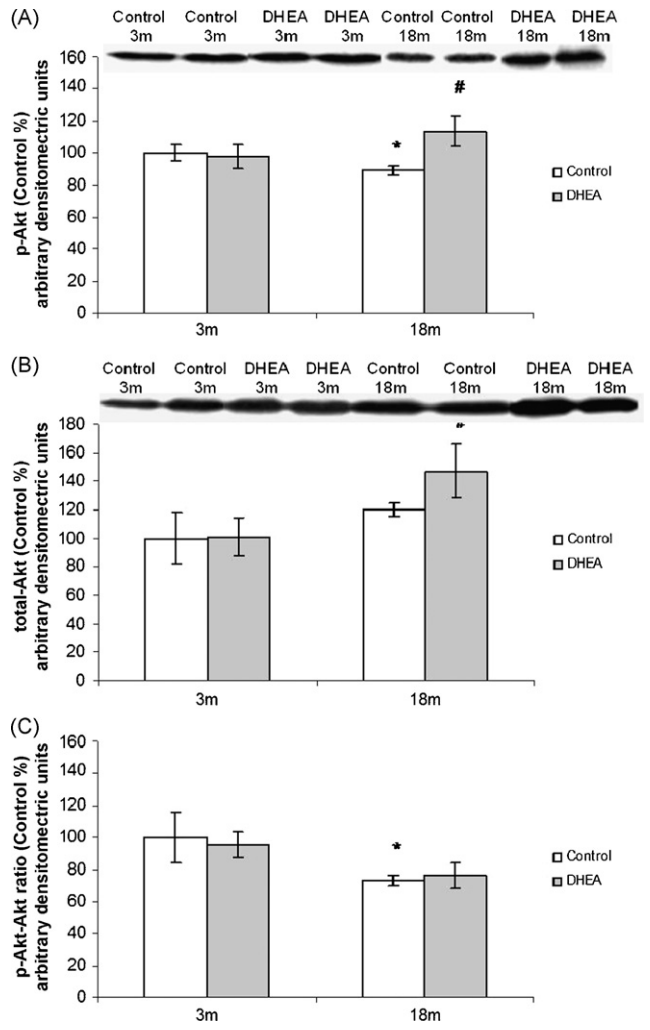

Fig. 3. Western blot analysis in hippocampus of 3 and 18 month-old rats after chronic DHEA treatment. (A) pAkt; (B) total-Akt; (C) pAkt/total-Akt ratio. $n = 4$–5 for each group. $^{*}P < 0.05$ in comparison with 3 month-old control group. $^{\#}P < 0.05$ in comparison with 18 month-old control group.

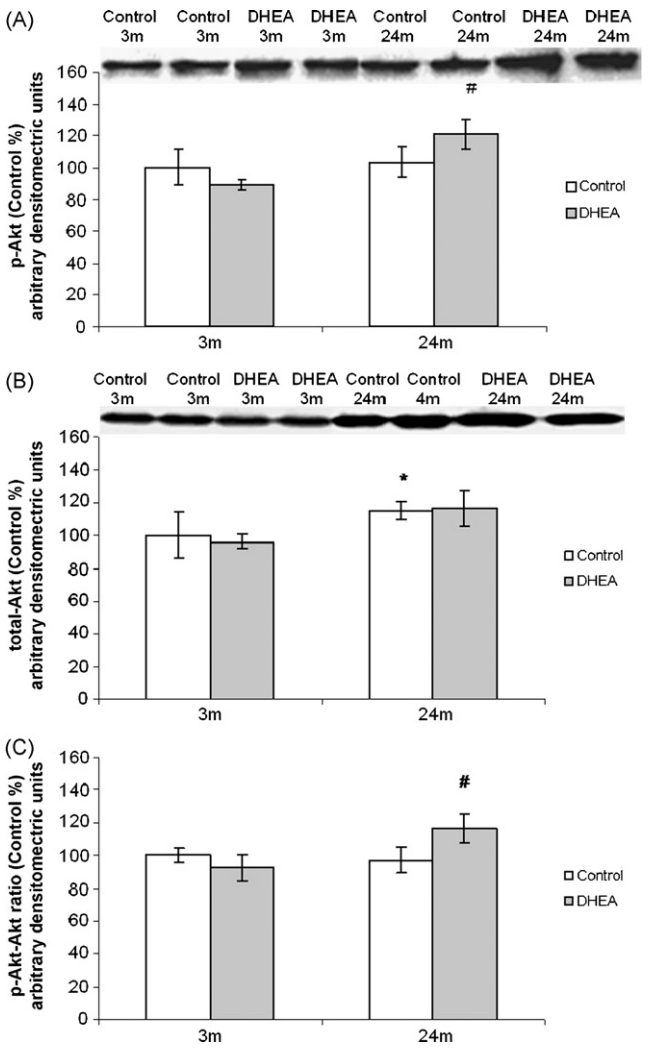

Fig. 4. Western blot analysis in hippocampus of 3 and 24 month-old rats after chronic DHEA treatment. (A) pAkt; (B) total-Akt; (C) pAkt/total-Akt ratio. $n = 4$ for each group. $^{*}P < 0.05$ in comparison with 3 month-old control group. $^{\#}P < 0.05$ in comparison with 24 month-old rats control group.

content was significantly increased in 24 month-old control group when compared to 3 month-old control group. DHEA treatment did not modify total-Akt in comparison to its control group (Fig. 4B). DHEA treatment increased pAkt/Akt ratio in hippocampus of 24 month-old group (Fig. 4C).

3.2. Hypothalamus

3.2.1. Acute treatment

PAkt and pAkt/Akt ratio significantly increased in hypothalamus only after 24 h of 50 mg/kg DHEA treatment, the highest tested dose (Fig. 5A and C). No change on Akt phosphorylation was found after 6 h of DHEA injection (data not shown). Additionally, total-Akt remained unchanged in hypothalamus after both acute experimental designs of DHEA treatment (after 6 or 24 h of DHEA administration in different concentrations).

3.2.2. Chronic treatment

Fig. 6 (3 and 24 month-old) shows data from Western blot analysis of Akt expression measured in the hypothalamus of rats after 5 weeks of a DHEA (10 mg/kg) administration once a week. There was no change of pAkt as well as in its total content in the hypothalamus of 3 month-old treated group when compared to its control.

There was no significant change in 18 month-old groups when compared to its control, independently of age or treatment, in pAkt, in total-Akt nor in pAkt/Akt ratio (data not shown).

There was a significant increase in 24 month-old control group when compared to 3 month-old control group, considering pAkt and also Akt content. DHEA treatment returned both pAkt and total-Akt levels in the hypothalamus of 24 month-old treated group to the levels of 3 month-old groups (Fig. 6A and B).

4. Discussion

The major outcome of this study was to demonstrate that in vivo DHEA treatment may modulate pAkt and total-Akt contents in the rat CNS. This effect was dose and time-dependent as well as age and tissue-dependent. The age variable also intervenes on Akt expression independently of DHEA treatment.

Aging is characterized by alterations in the chemistry and endocrinology of the brain that involves the synthesis and signaling pathways of neurotransmitters, neuropeptides, growth factors, and steroids [38]. Neurotrophic factors, growth factors and their receptors are abundant during development and decline with the age [39–41]. Therefore, these alterations could modify kinases activity which may directly contribute to age-dependent neural dysfunc-

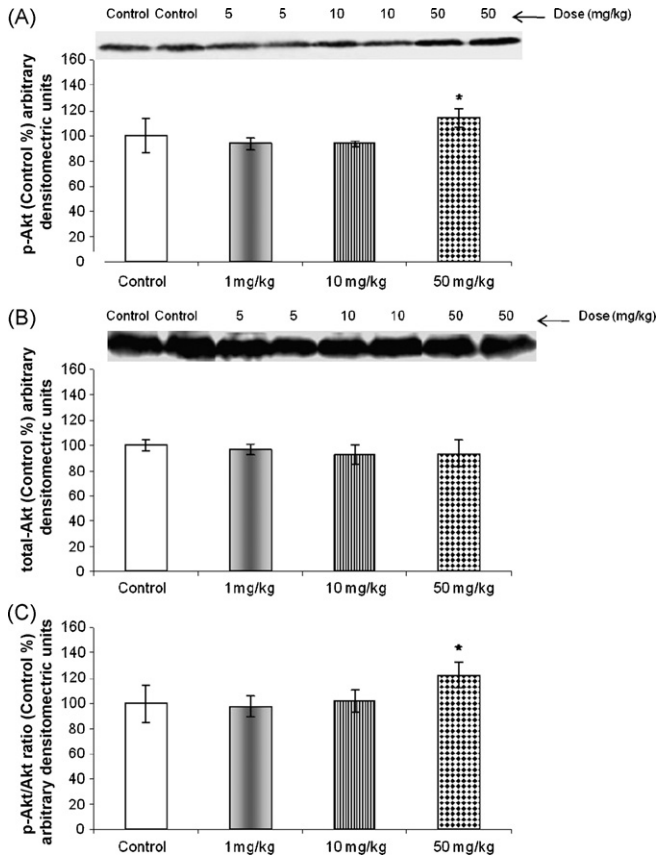

Fig. 5. Western blot analysis in hypothalamus after 24 h of DHEA treatment. (A) pAkt; (B) total-Akt; (C) pAkt/total-Akt ratio. $n = 4$ for each group. *Significantly different from all tested groups ($P < 0.05$).

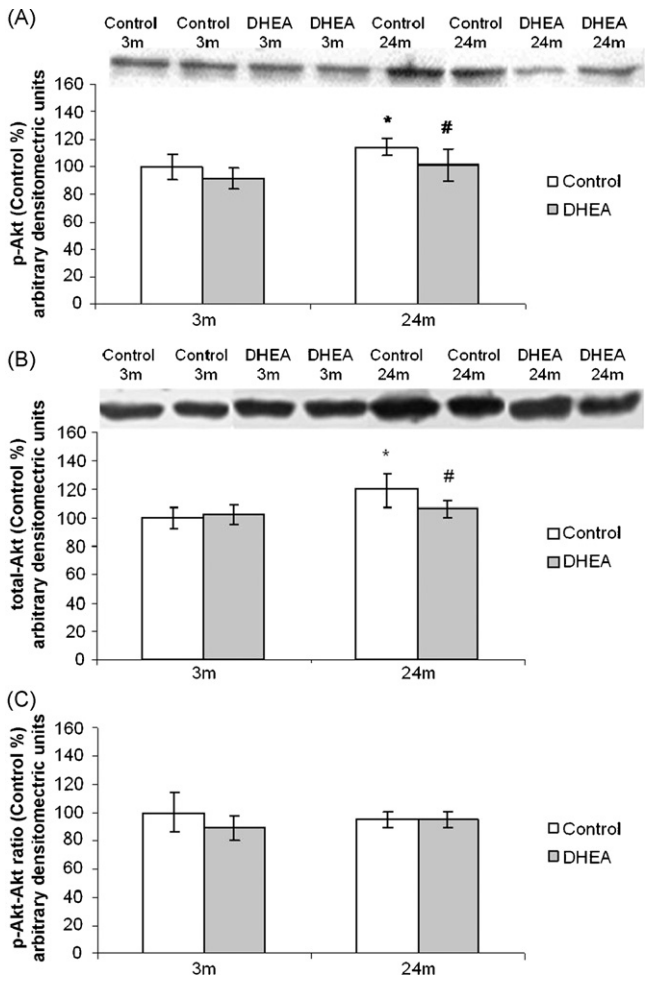

Fig. 6. Western blot analysis in hypothalamus of 3 and 24 month-old rats after chronic DHEA treatment. (A) pAkt; (B) total-Akt; (C) pAkt/total-Akt ratio. $n = 4$–5 for each group. *$P < 0.05$ in comparison with 3 month-old control group. #$P < 0.05$ in comparison with 24 month-old control group.

tions [41]. Akt has been well documented to promote neuronal survival. More recently, it has also been revealed as key mediator of several aspects of neurite outgrowth, including elongation, branching and caliber [42]. Akt signaling also could be modified with the aging process. In the present work it was shown a reduction of pAkt whereas total-Akt was unchanged in hippocampus of 18 month-olds (18 m) rats as compared to 3 month-old rats (3 m). This decreased pAkt expression leads to a reduction of 30% in the efficiency of Akt phosphorylation as observed by pAkt/Akt ratio. In spite of total-Akt level has been increased, pAkt expression was not altered in hippocampus of 24 month-old rats (in comparison to those 3 month-old). It seems that although there has been an enhancement in total-Akt content, it was ineffective to alter pAkt levels in hippocampus of older animals. Unlike hippocampus, in the hypothalamus of the oldest rats (24 months) the increase of total-Akt was followed by the increase in pAkt, while in 18 months there was not found significant alteration in the pAkt and total-Akt. Thus, hippocampus seems to be more susceptible to age effects on Akt modulation since this structure showed earlier effects than hypothalamus.

In accordance with our results regarding Akt phosphorylation reduction in 18 month-old group, in senescence-accelerated mouse (SAMP10) the phosphorylation of Akt (Ser^{473}) displayed a continuous decrease after 6 months in hippocampus, and when compared to control (SAMR1), aged mice showed significant reduced phosphorylation of Akt [28]. In contrast, accordingly to our results found regarding to the increase of total-Akt in hippocampus and in hypothalamus of the oldest rats (24 m), the number of Akt1/PKBalpha immunoreactive cells was increased in the hippocampal CA1 sector during aging processes [43] and was found an increase in activity of PKB/Akt in different CNS regions, including hippocampus of 80 and 96-week-old rats as compared to 3 and 12 week-old young rats [44]. Although Song et al. [44] did not found change in Akt content with aging, in our study we found an increase in both total-Akt and pAkt levels in hypothalamus 24 month-old rats. However, in hypothalamic insulin-stimulated, Akt phosphorylation decreased in 24 month-old rats as compared to 3 month-old rats [45].

These different results concerning Akt modulation and aging also have been found in other tissues and cells. While there are studies which have demonstrated a reduction on Akt activity, others have shown an increase in its phosphorylation status with aging process. An age-associated reduction of Akt activity was found in the kidney [46], pancreatic and hepatocytes cells [47,48] and skeletal muscle [49]. In addition, this reduction on Akt activity was linked with the lower survival as well as the maintenance of tissue functional integrity. On the other hand, studies have shown that Akt activity increases along with cellular senescence and its inhibition extends the lifespan of primary cultured endothelial cells [50].

Our study has shown different effects in Akt expression in CNS by chronic DHEA treatment according to age and structure. Chronic DHEA treatment restored the pAkt expression in hippocampus of 18 month-old rats. This effect seems to be secondary to enhanced total-Akt protein after DHEA treatment. Nevertheless, it was not found a significant difference on pAkt/Akt ratio In hippocampus

of 24 month-old rats, DHEA treatment also increased pAkt levels, and total-Akt was unchanged, increasing pAkt/Akt ratio, suggesting that DHEA might enhance the efficiency of Akt phosphorylation. Unlike hippocampus results, DHEA treatment decreased both total-Akt and pAkt levels in hypothalamus of 24 month-old rats. When the ratio of pAkt and total-Akt was calculated, no difference was detected in hypothalamus, suggesting that the pAkt decreased levels could be secondary to the loss of total-Akt.

Differently of aged DHEA-treated animals, it was not observed significant difference on total and pAkt levels in both regions studied after chronic DHEA administration at 3 month-old rats. In opposite to chronic treatment results, we reported that there was an increase in pAkt levels and pAkt/Akt ratio in both hippocampus and hypothalamus of 3 m rats after acutely DHEA administration with the highest tested dose (50 mg/kg). This effect was observed earlier in hippocampus, at 6 h, and it would be worthwhile to relate that, at this time, DHEA treatment could regulate pAkt levels without modify total-Akt. After 24 h of DHEA injection, the increase in pAkt could be secondary to the enhanced of total-Akt protein in hippocampus. However, there was also found a significance difference on pAkt/Akt ratio with the lowest DHEA doses (5 and 10 mg/kg) only after 24 h of DHEA injections. In hypothalamus, 50 mg/kg DHEA treatment increased pAkt without modify total-Akt. Thus, using different schedule paradigms (acute and chronic DHEA treatment) to male rats 3 month-old, DHEA had only short-term effects concerning to Akt modulation on the CNS.

DHEA may interfere directly on neurons and glial cells and also indirectly by intracrine effect by the formation of estrogens. Indeed, some DHEA effects are due to its conversion to estrogens [51,52]. Estradiol receptor interacts with IGF-1 activating PI3K/Akt cascade [53] and promoted an increase in the phosphorylation of Akt in hypothalamus as well as hippocampus of ovariectomized rats, and this last effect occurred after 6 and 12 h [54]. It is possible that estradiol and DHEA have similar signaling mechanisms since their analogous chemical structure and also by this steroid may be converted to estrogens.

Differential response to age and to DHEA treatment schedule found approaching total-Akt and pAkt levels could be related to different functions and physiological regulation of hippocampus and hypothalamus. Hippocampus seems to be more susceptible to age-related changes than hypothalamus as well as to be more susceptible to DHEA treatment in terms of Akt modulation. One point to consider is that we analyzed the entire hypothalamus, and separate hypothalamic nucleus may show distinct results, since hypothalamus is a very heterogeneous structure. DHEA seems to be closely involved in endocrine function, especially in reproductive and somatotrophic aspects [55–57]. Both regions are naturally affected by physiological aging, however hypothalamus may be associated with its essential role in the body homeostatic regulation, which should be maintained in aging. In this concern, hippocampal circuits, classically involved with learning and memory, are more disturbed with aging, which could be correlated with cognitive impairment age-related. Akt pathway is closely correlated to synaptic plasticity, learning and memory [58,59]. The more pronounced changes found in hippocampus might be associated with these functions.

Corroborating our results regarding to DHEA short-term administration, evidences from distinct in vitro approaches have already shown that DHEA-triggered Akt signaling could underlie some of its physiological effects. DHEA and its neuroprotective action has been shown to be mediated by activation of the phosphatidylinositol 3-kinase (PI3K)/Akt cascade in P19-N neurons [11] and PC12 cells [21]. DHEA can act as a survival factor for human and bovine endothelial cells by means of enhanced pAkt [60,61]. However, acute in vitro studies have described an inhibition in Akt phosphorylation by DHEA and DHEAS in hypothalamic neurons [62] and in embryonic forebrain [22]. DHEA induced apoptosis by down-regulating the Akt signaling pathway in GT1-7 hypothalamic neurons, when deprived of trophic support [62]. Recently, we have reported that acute (50 mg/kg) and chronic (10 mg/kg) DHEA treatment to 3 and 18 m rats, respectively, resulted in an elevation of pAkt/Akt ratio in myocardial as compared to control with the same dose and pattern of administration [32,35].

Most studies associating DHEA and Akt have been conducted in vitro, which do not reflect exactly in vivo neural environment. In addition, encephalic function is also under regulation by many factors, like cytokines, neuropeptides, growth factors, and others, which are absent in culture medium. The present work provides evidences about in vivo DHEA treatment as a modulator of Akt activity, an important prosurvival signaling pathway, in the CNS of adult and aged rats. This knowledge is crucial to understand the action mechanisms that could be involved in DHEA effects in the nervous system. New pharmacological strategies to prevent aging-related brain dysfunction would be developed focusing on DHEA administration and it would be interesting to follow downstream the signaling pathway of Akt. Changes in the endogenous capacity of the aged brain to synthesize and/or metabolize neuroactive steroids, as well as aged-associated alterations and signaling pathways modulation in the nervous system are important subjects for future investigation.

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