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Oxidative Derangement in Rat Synaptosomes Induced by Hyperglycaemia: Restorative Effect of Dehydroepiandrosterone Treatment

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ABSTRACT. Central nervous system damage in diabetes is caused by both cerebral atherosclerosis and the detrimental effect of chronic hyperglycaemia on nervous tissue. Hyperglycaemia is the primer of a series of cascade reactions causing overproduction of free radicals. There is increasing evidence that these reactive molecules contribute to neuronal tissue damage. Dehydroepiandrosterone (DHEA) has been reported to possess antioxidant properties. This study evaluates the oxidative status in the synaptosomal fraction isolated from the brain of streptozotocin-treated rats and the antioxidant effect of DHEA treatment on diabetic rats. Hydroxyl radical generation, hydrogen peroxide content, and the level of the reactive oxygen species was increased ($P < 0.05$) in synaptosomes isolated from streptozotocin-treated rats. The derangement of the oxidative status was confirmed by a low level of reduced glutathione and alpha-tocopherol. DHEA treatment (4 mg per day for 3 weeks, per os) protected the synaptosomes against oxidative damage: synaptosomes from diabetic DHEA-treated rats showed a significant decrease in reactive species ($P < 0.05$) and in the formation of end products of lipid peroxidation, evaluated in terms of fluorescent chromolipid ($P < 0.01$). Moreover, DHEA treatment restored the unsaturated fatty acid content of the membrane and the reduced glutathione and alpha-tocopherol levels to normal levels and restored membrane NaK-ATPase activity close to control levels. The results demonstrate that DHEA supplementation greatly reduces oxidative damage in synaptosomes isolated from diabetic rats and suggest that this neurosteroid may participate in protecting the integrity of synaptic membranes against hyperglycaemia-induced damage. BIOCHEM PHARMACOL **60**;3:389–395, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. dehydroepiandrosterone; synaptosomes; oxidative damage; antioxidant; hyperglycaemia; NaK-ATPase

Diabetes is a well-established risk factor for macrovascular disease: diabetic patients have been reported to have a poorer prognosis than non-diabetic patients after cerebrovascular accidents [1] or coronary artery disease [2]. However, damage to the central nervous system in diabetes is not only caused by cerebral atherosclerosis but also by the detrimental effects of chronic hyperglycaemia on nervous tissue [3]. Under experimental conditions, hyperglycaemia dramatically increases neuronal alterations and glial cell damage caused by temporary ischaemia [4]. Several lines of evidence indicate that the modified oxidative state induced by chronic hyperglycaemia [5, 6] may contribute to nervous tissue damage: free radical species impair both the central nervous system, attacking neurones and Schwann cells [7, 8] and the peripheral nerves [9]. Because of their high polyunsaturated lipid content, Schwann cells and axons are particularly sensitive to oxygen free radical damage: lipid peroxidation may increase cell membrane rigidity, damaging cell function. Oxidative stress seems to be involved in several neurological diseases, including Alzheimer's [10] and Parkinson's diseases [11], and recent evidence has indicated antioxidant treatment as providing a potential therapeutic approach to reducing the clinical manifestations of neurological disorders [12–14].

DHEA¶ and dehydroepiandrosterone sulphate (DHEA-S), the most abundant adrenal steroids in the blood, are synthesised *de novo* in the brain and are present in all regions of the human brain at concentrations higher than those in the plasma [15, 16]. These steroids play several roles in the central nervous system [17, 18]. Although DHEA, at very high (pharmacological) doses, actually induces oxidative stress in rats by acting as peroxisome

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¶ *Abbreviations:* DHEA, dehydroepiandrosterone; STZ, streptozotocin; ROS, reactive oxygen species; LDH, lactate dehydrogenase; $HO^{\cdot}$; hydroxyl radical; DCFH-DA, 2′,7′-dichlorofluorescin diacetate; and H_2O_2, hydrogen peroxide.

proliferator [19, 20], evidence indicates that, at concentrations slightly above those found in human tissues, it possesses a multitargeted antioxidant effect [21–24] and prevents tissue damage induced by hyperglycaemia [25–27].

The oxidative status of synaptosomes isolated from STZ-treated rats was studied herein, as was the effect of chronic oral DHEA treatment on oxidative brain damage produced by hyperglycaemia. Synaptosomes, the isolated terminal portions of axons that behave as metabolically autonomous mini-cells, provide a good experimental model to evaluate nervous degenerative processes and peroxidative events in cerebral cells [28]. The results show a positive effect of DHEA treatment against oxidative damage induced by chronic hyperglycaemia in rat brain synaptosomes.

MATERIALS AND METHODS

Material

Adenosine triphosphate, dehydroisoandrosterone (5-androsten-3β-ol-17-one, dehydroepiandrosterone), streptozotocin, (+)-α-tocopherol, NADH, reduced and oxidised glutathione, dichlorofluorescin, 2′, 7′-dichlorofluorescin diacetate, mono- and polyunsaturated fatty acid methyl ester standards, and the internal standard heptadecanoic acid (17:0) were purchased from Sigma Chemical Co. Folin–Ciocalteu phenol reagent, hydrogen peroxide, and EDTA were purchased from Merck.

Animals and Treatments

Male Wistar rats (Harlan Nossan) weighing 250–280 g (body wt) were used. The animals were housed and cared for in compliance with the Italian Ministry of Health Guidelines and with the Principles of Laboratory Animal Care (NIH no. 85-23, revised 1985) and were provided with Piccioni pellet diet (no. 48, Gessate Milanese, Italy) and water *ad lib*. Before starting the experimental protocols, the rats were weighed and randomly divided into four groups of 7–10 animals: control, DHEA alone, STZ alone, and STZ plus DHEA. Two groups were treated with a single dose of STZ (50 mg/kg body wt) injected into the tail vein after light ethyl ether anaesthesia. Streptozotocin was diluted in 0.05 M pH 4.5 citrate buffer to obtain a final volume of 0.25 mL/100 g body wt per injection. STZ is rapidly eliminated from the body, with about 80% appearing in the urine within 6 hr [5]. Three days after the injection, glycaemia was measured; only those rats with blood glucose levels above 20 mmol/L were included in the experimental protocols. The other two groups of rats received the same volume of physiological solution in the tail vein. On the fourth day postinjection, one group of hyperglycaemic rats and one group of control rats began oral treatment with DHEA, by gastric intubation, at a single daily dose (4 mg in 0.5 mL of mineral oil) for 21 consecutive days; the other two groups received vehicle alone. On day 22 the rats were weighed, killed by aortic exsanguination, and decapitated; blood was collected for plasma and serum isolation. The experimental protocol was determined on the basis of previous works, which demonstrated that DHEA at 4 mg/day for 21 days protected tissues of STZ rats from lipid peroxidation [27].

Isolation of Synaptosomal Membranes

Following the method described by Hajos [29], cerebral hemispheres were dropped into ice-cold isolation medium (0.3 M sucrose) and immediately weighed and finely chopped. The tissue was homogenised (10%, w/v) in a Potter-Elvejhem homogeniser with 12 strokes of the glass pestle; the homogenate was centrifuged by sequential centrifugation. The final synaptosomal pellet from each rat was taken up in isolation medium to a final volume of 4–4.5 mL (~5 mg prot/mL).

Biochemical Determinations

Glycaemia was evaluated with *o*-toluidine reagent on fresh plasma (Sigma kit, cat no. 635). On fresh synaptosomal fraction, isolated from both control and treated rats, the following determinations were made: protein content was measured by the method of Lowry *et al.* [30]; lactate dehydrogenase (LDH, EC 1.1.1.27) was evaluated spectrophotometrically at 340 nm by Lai and Clark's method [31]; DHEA concentration was measured in plasma by the RIA (radioimmunoassay) method after extraction and chromatographic procedures [32]; reduced glutathione and total glutathione content was determined by the method of Owens and Belcher [33]. Aliquots of the synaptosomal fraction were prepared in trichloroacetic (TCA)–EDTA (10%: 10 mM, v/v), centrifuged, and the supernatant used. A mixture was directly prepared in the cuvette: 0.05 M Na-phosphate buffer, pH 7.0; 1 mM EDTA, pH 7.0; 10 mM 5,5′-dithiobis(2-nitrobenzoic) acid (DTNB) plus an aliquot of the sample. This was monitored at 412 nm for 2 min (reduced glutathione), after which suitable diluted glutathione reductase and NADPH were added (total glutathione). Alpha-tocopherol was assayed by the method described by Burton *et al.* [34]: after extraction of the sample (0.5 mL aliquot) with 1 mL *n*-heptane and brief centrifugation, the heptane phase was collected for HPLC analysis. A Supercosil-Lc-Si column (25 cm × 4.6 mm, Supelco Inc.) was used, the mobile phase being *n*-hexane–isopropanol (99:1, v/v) and the flow rate 2.0 mL/min; the fluorescence detector was set to 298 nm excitation and 325 nm emission.

The end products of lipid peroxidation were evaluated in terms of fluorescent chromolipids. Total lipids were extracted by the method of Folch *et al.* [35]. The fluorescence intensity of the samples was evaluated at 360 nm excitation and 430 nm emission by the method of Esterbauer *et al.* [36].

The technique used for measuring the hydroxyl radical ($HO^{\cdot}$) was that described by Halliwell [37]. Hydroxyl radicals attack the sugar deoxyribose, degrading it into

TABLE 1. Glucose and DHEA levels in plasma obtained from control, STZ, DHEA, and STZ plus DHEA rats

	Control	STZ	DHEA	STZ + DHEA
Glucose (mmol/L)	7.7 ± 1.7	31.7 ± 2.5*	7.5 ± 1.0	30.2 ± 2.6*
DHEA (ng/mL plasma)	1.81 ± 0.51	1.21 ± 0.72	4.32 ± 2.01	3.75 ± 2.02

Values are means of 7–10 rats ± SD.
*Statistical significance: $P < 0.001$ vs control.

fragments, some of which react with thiobarbituric acid, colouring a solution pink. The deoxyribose assay gives an approximate indication of the ability of a compound to interfere with iron ion-dependent site-specific Fenton chemistry. Deoxyribose (2.8 mM) degradation, in the presence of ascorbate (100 μM), $FeCl_3$ (100 μM), and EDTA (10 μM) plus sample (~1 mg prot), was measured by the thiobarbituric acid test [37]. The absorbance was at 532 nm. In order to evaluate thiobarbituric acid-reactive substance (TBARS) formation produced by the synaptosomes, controls without deoxyribose were run; the values obtained were subtracted from those for the samples.

ROS were measured using probe DCFH-DA. DCFH-DA is a stable, non-fluorescent molecule that readily crosses the cell membrane and is hydrolysed by intracellular esterases to non-fluorescent 2′,7′-dichlorofluorescin (DCFH), which is rapidly oxidised, in the presence of peroxides, to highly fluorescent 2′,7′-dichlorofluorescein (DCF). The DCF is measured fluorimetrically [38]. To rule out the possibility that different esterase activities in the different groups might invalidate the ROS assay, an already oxidised probe was used as control compound. In separate experiments, different concentrations of H_2O_2 (1–10 μM) were added to the synaptosomal fractions incubated with DCFH-DA, and ROS levels were measured.

The generation of H_2O_2 was monitored by adding peroxidase from horseradish and acetylated ferrocytochrome *c* to the sample [39]. The synaptosomal H_2O_2 release was measured as the increase in the acetylated ferrocytochrome *c* oxidation rate and monitored at 550 nm minus 540 nm using an absorption coefficient of 19.9 mM^{-1} cm^{-1}, as described by Zoccarato *et al.* [40].

NaK-ATPase (Na^+, K^+-ATPase Mg^{2+}-dependent ATP phosphohydrolase, E.C. 3.6.1.3) activity was assayed in a medium containing imidazolo–HCl buffer pH 7.4, 120 mM NaCl, 10 mM KCl, 5 mM $MgCl_2$, and 4 mM ATP. The reaction was started by adding 25 μg of synaptosomal membrane protein and carried out as described by Shallom and Katyare [41].

Fatty Acid Analysis from Synaptosomes

Fatty acid content of synaptosomal lipids was determined by GLC. Synaptosomal lipids were extracted by the method of Folch *et al.* [35], methylated with 5 mL NaOH in 0.5 N MetOH, and hydrolysed with 5 mL of 6N HCl [42]. As internal standard, heptadecanoic acid (17:0) was added to the solution. The mixture was vortexed, centrifuged, and the $CHCl_3$ phase removed and evaporated under vacuum. The samples were redissolved in 300 μL $CHCl_3$. GLC was performed by injecting 3 μL of sample into a Perkin Elmer-Sigma 300 Dual Chromatograph equipped with flame ionisation detector. Separations were performed with a WCOT fused silica (25 m × 0.25 mm) column coated with CP-SIL 5CB, DF-0.25 (Chromopack, The Netherlands). The temperature program was 200° for 30 min, increasing linearly to 220° at 5°/min, then constant for 30 min. The peaks were identified by comparison with the retention times of methyl ester fatty acid standards. Quantitation of fatty acids was by comparison with the internal standard (17:0).

Statistical Analysis of Data

Values reported are means of 7–10 animals per group ± SD. Differences between means were analysed for significance using the one-way ANOVA test with the Bonferroni *post hoc* test [43].

RESULTS

At the start of the experimental protocol, body weight was in the 270 ± 10 gram range. After three weeks, the diabetic rats had undergone a marked weight loss. The body weight of the groups treated with STZ was lower than controls ($P < 0.001$). The glucose level increased drastically in STZ-treated rats three days after STZ injection (data not reported). Hyperglycaemia was reconfirmed at the end of the experiments in STZ-treated rats and was not modified by DHEA treatment throughout the experiment (Table 1). During DHEA treatment, the plasma level of the steroid reached values similar to those found in normal humans [44].

Oxidative State in Synaptosomes

The oxidative state of the synaptosomal fraction of the STZ-treated rats is shown in Table 2. Both the H_2O_2 level and $HO^{\cdot}$ radicals increased markedly in STZ rats compared to controls; both measures returned to control levels in the synaptosomes of DHEA-treated STZ animals. The ROS level was also markedly increased in diabetic rats while, in rats treated with STZ plus DHEA, it was not different from that of controls. In the reaction to determine ROS levels, DCFH-DA was converted to DCF by intracellular esterase; to rule out any difference in esterase activity in the different

TABLE 2. Oxidative state evaluated in terms of HO˙, ROS, and H_2O_2 levels in brain synaptosomes isolated from control, STZ, DHEA, and STZ plus DHEA rats

	Control	STZ	DHEA	STZ + DHEA
HO˙ (Abs 523 nm)	1.03 ± 0.05	1.27 ± 0.06*	1.09 ± 0.12	1.07 ± 0.07†‡
ROS (U.F./mg prot)	0.96 ± 0.12	1.93 ± 0.39*	0.82 ± 0.08	0.90 ± 0.13†‡
H_2O_2 (pmol/min/mg prot)	17.92 ± 1.62	21.72 ± 1.24*	16.95 ± 1.02	18.35 ± 1.02†‡

Values are means of 7–10 rats ± SD. U.F., units of fluorescence.
Statistical significance: *$P < 0.05$ vs control; †$P < 0.05$ vs STZ; and ‡ not significant vs DHEA.

groups, H_2O_2 was utilised as specific probe. The fluorescent DCF value, formed following incubation with H_2O_2, was not different in the different groups (data not reported). In synaptosomes isolated from hyperglycaemic rats, H_2O_2 production was higher than in the control group; this increase was reduced by DHEA treatment.

Non-enzymatic Defences against Free Radicals

The levels of the physiological antioxidant barriers detected, namely reduced glutathione and alpha-tocopherol, are shown in Table 3. Reduced glutathione and alpha-tocopherol levels were below normal values in the synaptosomes of STZ rats, as has been reported for other tissues of diabetic rats [12]. DHEA treatment in STZ rats was able to prevent the drop in both reduced glutathione and α-tocopherol levels: in the DHEA-treated diabetic rats, both antioxidants were comparable to controls. Total glutathione levels in the STZ-treated rats were unmodified compared to control values (Table 3).

Lipid Peroxidation and Membrane Integrity

In synaptosomes isolated from STZ-treated rats, oxidative insult induced a degradation of membrane polyunsaturated fatty acids, with an accumulation of the end products of the lipid peroxidation process that was measured in terms of fluorescent chromolipids. The oxidative state improved when DHEA was given to the rats: Figure 1 shows a marked increase in the number of fluorescent chromolipids in STZ rats, whereas this value was significantly lower in those also treated for three weeks with DHEA.

The integrity of the synaptosomal membrane was evaluated by measuring both LDH release [45] and NaK-ATPase activity. LDH appeared up-modified in diabetic rats, while when DHEA was given to STZ rats, LDH increased compared to controls but was significantly lower than STZ-treated rats (Fig. 2). Moreover, ATP hydrolysis in diabetic rats was markedly reduced compared to controls, although it did increase significantly after DHEA treatment. Thus, DHEA partially protected NaK-ATPase activity of the synaptosomal membranes (Fig. 2).

The fatty acid content of synaptosomal membranes from DHEA rats with STZ-induced diabetes was also determined. In line with other reports on heart and liver [46, 47], our results show a marked decrease in arachidonic and oleic acid contents in synaptosomes of STZ-treated rats (Fig. 3). When DHEA treatment was administered to STZ rats, the level of these fatty acids was returned to control values.

DISCUSSION

This work is one of a series of studies showing that chronic hyperglycaemia causes an imbalance in the oxidative status of the nervous tissue and that the resulting free radicals damage the presynaptic region of the neurones through a peroxidative mechanism. Under normal conditions, the generation of free radicals or of active species in the brain, as in other tissues, is maintained at extremely low levels [48]. Here, we show that, in synaptosomes isolated from STZ-treated rats, HO˙ radicals are increased, as are the levels of H_2O_2 and of ROS. The high sugar level functions as a catalyst of oxidative chemical reactions involving glucose, through glycation, glyco-oxidation, and auto-oxidative glycation [6, 49].

The derangement of the oxidative state in synaptosomes is here confirmed by the low level of non-enzymatic antioxidants, the endogenous reduced glutathione, and the exogenous alpha-tocopherol. This condition of oxidative imbalance induced by hyperglycaemia leads to peroxidation of lipid membranes, indicated by the increase in the end products of lipid peroxidation, which can cause changes in the physical properties, i.e., permeability and fluidity, of the nerve terminals. The importance of antioxidant treatment

TABLE 3. Reduced glutathione, total glutathione, and α-tocopherol levels in brain synaptosomes isolated from control, STZ, DHEA, and STZ plus DHEA rats

	Control	STZ	DHEA	STZ + DHEA
Reduced glutathione (nmol/mg prot)	0.012 ± 0.001	0.004 ± 0.001*	0.010 ± 0.001	0.008 ± 0.001†
Total glutathione (nmol/mg prot)	0.221 ± 0.011	0.183 ± 0.016	0.214 ± 0.025	0.225 ± 0.036
α-Tocopherol (pmol/mg prot)	498.74 ± 47.14	147.81 ± 4.42*	566.41 ± 41.03	493.70 ± 83.20†

Values are means of 7–10 rats ± SD.
Statistical significance: *$P < 0.05$ vs control; †$P < 0.05$ vs STZ.

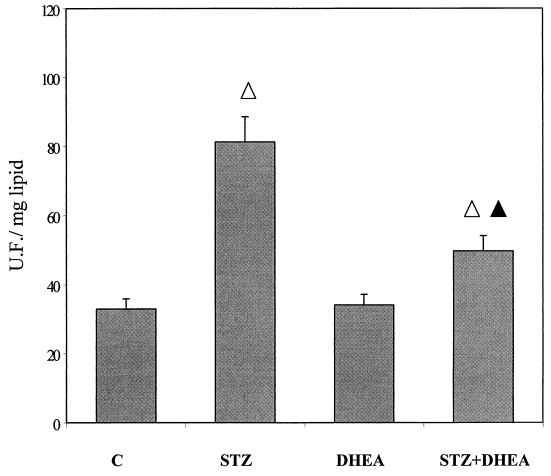

FIG. 1. Level of fluorescent chromolipids, expressed as units of fluorescence (U.F.), in brain synaptosomes isolated from control, STZ, DHEA, and STZ plus DHEA rats. Values are means of 7–10 rats ± SD. Statistical significance: Δ, $P < 0.05$ vs control; ▲, $P < 0.01$ vs STZ.

in the prevention of neuronal damage has recently been emphasised [12, 13, 50]. Here, we show the beneficial effect of DHEA treatment in preventing lipid membrane peroxidation caused by hyperglycaemia. In isolated STZ synaptosomes, $HO^{\cdot}$ levels, as well as the production of H_2O_2 and ROS and the end products of lipid peroxidation, are markedly reduced by DHEA, suggesting that it may be an excellent scavenger, and thus an antioxidant, in the brain.

Indeed, the possibility that inserting DHEA into lipid membranes makes them more resistant to oxidative stress should be considered. The intercalation of DHEA into lipid membranes has been suggested as the mechanism responsible for the change in shape induced *in vitro* by DHEA in human red blood cells [51]. Moreover, DHEA has been reported to change the fatty acid composition of mitochondrial membrane phospholipids in rats [52]. In our experiments, we observed a slighter derangement of the polyunsaturated fatty acid content of membranes in DHEA-treated rats compared to STZ-alone-treated rats. Thus, another cause of free radical damage, i.e. 4-hydroxynonenal production from lipid membrane breakdown, is reduced. In fact, these aldehydes are highly reactive and may be a second messenger that disseminates initial free radical events [53]. The restoration of non-enzymatic antioxidant barriers (reduced glutathione and alpha-tocopherol) to control levels confirms the protective effect of DHEA against oxidative derangement. While other studies have shown an improvement in the electrophysiological deficit following antioxidant treatment in experimental diabetic neuropathy [9], none of these has measured lipid peroxidation products. Here, we show that DHEA treatment prevents the accumulation of fluorescent chromolipid induced by hyperglycaemia.

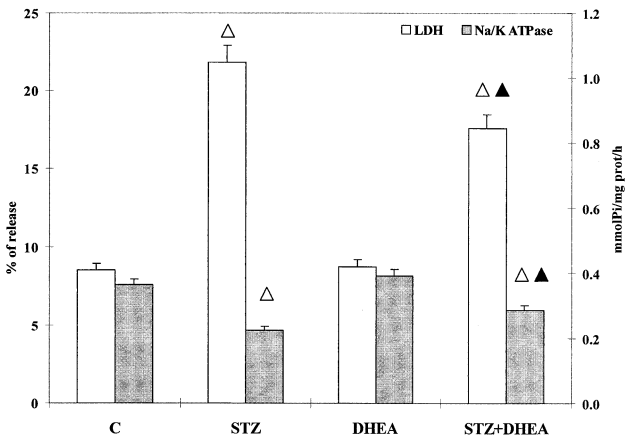

FIG. 2. LDH release and NaK-ATPase activity in brain synaptosomes isolated from control, STZ, DHEA, and STZ plus DHEA rats. Values and statistical significance are as in Fig. Leg. 1.

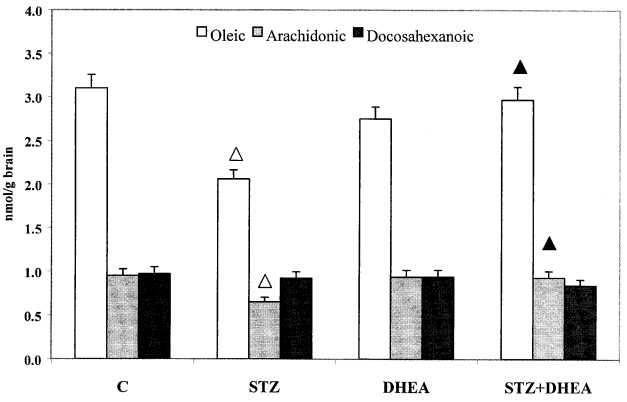

FIG. 3. Fatty acid unsaturated content in brain synaptosomes isolated from control, STZ, DHEA, and STZ plus DHEA rats. Values and statistical significance are as in Fig. Leg. 1.

The improved membrane integrity when DHEA is given to hyperglycaemic rats can also be argued from the improved NaK-ATPase activity that is observed. The fall in NaK-ATPase activity in diabetes [54, 55] has been attributed to the increase in lipid peroxide products and to increased polyol pathway activity coupled with reduced *myo*-inositol [56]: both mechanisms are counteracted by DHEA. In fact, DHEA treatment partially suppresses the increase in brain aldose reductase activity observed in diabetes [27]. Since NaK-ATPase plays a crucial role in neuronal functions, maintaining the ionic gradient necessary for both cell metabolism and nervous excitability, the saving effect of DHEA seems crucial for efficiently protecting the synapses from membrane dysfunction induced by hyperglycaemia.

The derangement of synaptic membrane integrity and function induced by diabetes is confirmed by the increase of LDH release. However, LDH output as well as the drop in NaK-ATPase activity are only partially counteracted by DHEA treatment in face of the absence of any increased load of oxygen radicals. This would suggest that a component causing cell death in diabetic animals could be due to

other sources, such as energy failure, than the formation of free radicals.

The neurosteroid DHEA has been reported to have a powerful ameliorative effect on glutamate-induced neurotoxicity in cerebral ischaemia, via a mechanism that is not yet evident [24]. We suggest that this effect of DHEA may be dependent on its antioxidant properties, since it has been demonstrated that aldehydic products derived from lipid oxidation impair glutamate transport in cortical synaptosomes and cause excito-toxic synaptic degeneration [57].

In conclusion, we have shown that DHEA supplementation protects synaptosomes isolated from STZ-diabetic rats against oxidative damage. This finding, together with previous studies in cell cultures and animals, points the way towards an additional therapeutic approach to neuronal failure in diabetes.

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References

1. Berger L and Hakim M, The association of hyperglycemia with cerebral edema in stroke. *Stroke* **17:** 865–871, 1986.
2. Barzilay JI, Kronmal RA, Bittner V, Eaker E and Foster ED, Coronary artery disease in diabetic and nondiabetic patients with lower extremity arterial disease: A report from the Coronary Artery Surgery Study Registry. *Am Heart J* **135:** 1055–1062, 1998.
3. Swidan SZ and Montgomery PA, Effect of blood glucose concentrations on the development of chronic complications of diabetes mellitus. *Pharmacotherapy* **18:** 961–972, 1998.
4. Li PA, Gisselsson L, Keuker J, Vogel J, Smith ML, Kuschinsky W and Siesjo BK, Hyperglycemia-exaggerated ischemic brain damage following 30 min of middle cerebral artery occlusion is not due to capillary obstruction. *Brain Res* **804:** 36–44, 1998.
5. Oberley LW, Free radicals and diabetes. *Free Radic Biol Med* **5:** 113–124, 1988.
6. Hunt JV, Glucose: Chemistry and atherosclerosis in diabetes mellitus. In: *Free Radicals in the Environment, Medicine and Toxicology* (Eds. Nohl H, Esterbauer H and Rice-Evans C), pp. 137–162. Richelieu Press, London, 1994.
7. Kumar JS and Menon VP, Effect of diabetes on levels of lipid peroxides and glycolipids in rat brain. *Metabolism* **42:** 1435–1439, 1993.
8. Kawai N, Keep RP, Betz AL and Nagao S, Hyperglycemia induces progressive changes in the cerebral microvasculature and blood–brain barrier transport during focal cerebral ischemia. *Acta Neurochir Suppl(Wien)* **71:** 219–221, 1998.
9. Karasu C, Dewhurst M, Stevens EJ and Tomlinson DR, Effect of anti-oxidant treatment on sciatic nerve dysfunction in streptozotocin-diabetic rats; Comparison with essential fatty acid. *Diabetologia* **38:** 129–134, 1995.
10. Markesbery WR and Lovell MA, Four-hydroxynonenal, a product of lipid peroxidation, is increased in the brain in Alzheimer's disease. *Neurobiol Aging* **19:** 33–36, 1998.
11. Selley ML, (*E*)-4-hydroxy-2-nonenal may be involved in the pathogenesis of Parkinson's disease. *Free Radic Biol Med* **25:** 169–174, 1998.
12. Low PA, Nickander KK and Tritschler HJ, The roles of oxidative stress and antioxidant treatment in experimental diabetic neuropathy. *Diabetes* **46:** S38–42, 1997.
13. Urano S, Asai Y, Makabe S, Matsuo M, Izumiyama N, Ohtsubo K and Endo T, Oxidative injury of synapse and alteration of antioxidative defence systems in rats, and its prevention by vitamin E. *Eur J Biochem* **245:** 64–70, 1997.
14. Vatassary GT, Vitamin E and other endogenous antioxidants in the central nervous system. *Geriatrics* **53:** S25–S27, 1998.
15. Baulieu EE, Robel P, Vatier O, Haug A, Le Goascogne C and Borreau E, In: *Receptor-Receptor Interaction, A New Intramembrane Integrative Mechanism* (Eds. Fuxe K and Agnati LF), pp. 89–194. Macmillan, Basingstoke, 1987.
16. Zwain IH and Yen SS, Dehydroepiandrosterone: Biosynthesis and metabolism in the brain. *Endocrinology* **140:** 880–887, 1999.
17. Bologa L, Sharma J and Roberts E, Dehydroepiandrosterone and its sulfated derivative reduce neuronal death and enhance astrocytic differentiation in brain cell cultures. *J Neurosci Res* **17:** 225–234, 1987.
18. Majewska MD, Neuronal actions of dehydroepiandrosterone. Possible roles in brain development, aging, memory, and affect. *Ann N Y Acad Sci* **774:** 111–120, 1995.
19. Swierczynski J, Bannasch P and Mayer D, Increase of lipid peroxidation in rat liver microsomes by dehydroepiandrosterone feeding. *Biochim Biophys Acta* **1315:** 193–198, 1996.
20. Waxman DJ, Role of metabolism in the activation of dehydroepiandrosterone as a peroxisome proliferator. *J Endocrinol* **150** Suppl: S129–S147, 1996.
21. Arad Y, Badimon JJ, Badimon L, Hembree WC and Ginsberg HN, Dehydroepiandrosterone feeding prevents aortic fatty streak formation and cholesterol accumulation in cholesterol-fed rabbit. *Arteriosclerosis* **9:** 159–166, 1989.
22. Aragno M, Tamagno E, Boccuzzi G, Brignardello E, Chiarpotto E, Pizzini A and Danni O, Dehydroepiandrosterone pretreatment protects rats against the pro-oxidant and necrogenic effects of carbon tetrachloride. *Biochem Pharmacol* **46:** 1689–1694, 1993.
23. Boccuzzi G, Aragno M, Seccia M, Brignardello E, Tamagno E, Albano E, Danni O and Bellomo G, Protective effect of dehydroepiandrosterone against copper-induced lipid peroxidation in the rat. *Free Radic Biol Med* **22:** 1289–1294, 1997.
24. Kimonides VG, Khatibi NH, Svendsen CN, Sofroniew MV and Herbert J. Dehydroepiandrosterone (DHEA) and DHEA-S (DHEA-S) protect hippocampal neurones against excitatory amino acid-induced neurotoxicity. *Proc Natl Acad Sci USA* **17:** 1852–1857, 1998.
25. Aragno M, Brignardello E, Tamagno E, Gatto V, Danni O and Boccuzzi G, Dehydroepiandrosterone administration prevents the oxidative damage induced by acute hyperglycemia in rats. *J Endocrinol* **158:** 21–26, 1997.
26. Brignardello E, Beltramo E, Molinatti PA, Aragno M, Gatto V, Tamagno E, Danni O, Porta M and Boccuzzi G, Dehydroepiandrosterone protects bovine retinal capillary pericytes against glucose toxicity. *J Endocrinol* **158:** 21–26, 1998.
27. Aragno M, Tamagno E, Gatto V, Brignardello E, Parola S, Danni O and Boccuzzi G, Dehydroepiandrosterone protects tissues of streptozotocin-treated rats against oxidative stress. *Free Radic Biol Med* **26:** 1467–1474, 1999.
28. Whittaker VP, Membranes in synaptic function. In: *Cell Membranes. Biochemistry, Cell Biology and Pathology* (Eds. Weissmann G and Claiborne R), pp. 167–175. Hospital Practice Publishing Co., Inc., New York, 1975.
29. Hajos F, An improved method for the preparation of synaptosomal fractions in high purity. *Brain Res* **93:** 485–489, 1975.
30. Lowry OH, Rosebrough NJ, Farr AL and Randall RJ, Protein measurement with the folin phenol reagent. *J Biol Chem* **193:** 265–275, 1951.

31. Lai JC and Clark JB, Preparation and properties of mitochondria derived from synaptosomes. *Biochem J* **154:** 423–432, 1976.
32. Boccuzzi G, Brignardello E, Massobrio M and Bonino L, Breast duct fluid dehydroepiandrosterone sulfate in fibrocystic disease. *Eur J Cancer Clin Oncol* **23:** 1099–1102, 1987.
33. Owens CW and Belcher RV, A colorimetric micro-method for the determination of glutathione. *Biochem J* **94:** 705–711, 1965.
34. Burton GW, Webb A and Ingold KU, A mild, rapid and efficient method of lipid extraction for use in determining vitamin E/lipid ratios. *Lipids* **20:** 29–39, 1985.
35. Folch J, Lees M and Sloane Stanley GH, A simple method for the isolation and purification of total lipids from animals tissues. *J Biol Chem* **226:** 497–509, 1957.
36. Esterbauer H, Koller E, Slee RG and Koster JF, Possible involvement of the lipid peroxidation product 4-hydroxynonenal in the formation of fluorescent chromolipids. *Biochem J* **239:** 405–409, 1986.
37. Halliwell B, How to characterize a biological antioxidant. *Free Radic Res Commun* **9:** 1–32, 1990.
38. Ravindranath V, Animal models and molecular markers for cerebral ischemia–reperfusion injury in brain. In: *Methods in Enzymology* (Ed. Packer L) Vol. 233, pp. 610–619. Academic Press, New York, 1994.
39. Zoccarato F, Valente M and Alexandre A, Identification of an NADH plus iron dependent, Ca^{2+} activated hydrogen peroxide production in synaptosomes. *Biochim Biophys Acta* **1176:** 208–214, 1993.
40. Zoccarato F, Deana R, Cavallini L, and Alexandre A, Generation of hydrogen peroxide by cerebral-cortex synaptosomes. Stimulation by ionomycin and plasma-membrane depolarization. *Eur J Biochem* **180:** 473–478, 1989.
41. Shallom JM and Katyare SS, Altered synaptosomal ATPase activity in rat brain following prolonged *in vivo* treatment with nicotine. *Biochem Pharmacol* **34:** 3445–3449, 1985.
42. Morgan TE, Hanahan DJ and Ekholm J, A rapid method for deacylation of phospholipids and neutral lipids. *Fed Proc* **22:** 414–417, 1963.
43. Matthews DE and Farewell VT, Linear regression models for medical data. In: *Using and Understanding Medical Statistics, 2nd Revised Ed.*, pp. 134–140. Karger, Basel, 1988.
44. Vermeulen A, Dehydroepiandrosterone sulfate and aging. *Ann N Y Acad Sci* **774:** 121–127, 1995.
45. Koh J and Choi D, Quantitative determination of glutamate mediated cortical neuronal injury in cell culture by lactate dehydrogenase efflux assay. *J Neurosci Methods* **20:** 83–90, 1987.
46. Shin CS, Lee MK, Park KS, Kim SY, Cho BY, Lee HK, Koh CS and Min MK, Insulin restores fatty acid composition earlier in liver microsomes than erythrocyte membranes in streptozotocin-induced diabetic rats. *Diabetes Res Clin Pract* **29:** 93–8, 1995.
47. Kuwahara Y, Yanagishita T, Konno N and Katagiri T, Changes in microsomal membrane phospholipids and fatty acids and in activities of membrane-bound enzyme in diabetic rat heart. *Basic Res Cardiol* **92:** 214–222, 1997.
48. Braughler JM and Hall ED, Central nervous system trauma and stroke. I. Biochemical consideration for oxygen radical formation and lipid peroxidation. *Free Radic Biol Med* **6:** 289–301, 1989.
49. Baynes JW, Role of oxidative stress in development of complications in diabetes. *Diabetes* **40:** 405–412, 1991.
50. Cameron NE, Cotter MA and Maxfield EK, Anti-oxidant treatment prevents the development of peripheral nerve dysfunction in streptozotocin-diabetic rats. *Diabetologia* **36:** 299–304, 1993.
51. Hu ML, Ng HP and Shih MK, Hemolytic effects of dehydroepiandrosterone *in vitro*. *Life Sci* **61:** 2137–2142, 1997.
52. Mohan P and Clearly M, Short-term effects of dehydroepiandrosterone treatment in rats on mitochondrial respiration. *J Nutr* **121:** 240–250, 1991.
53. Esterbauer H, Schaur RJ and Zollner H, Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes. *Free Radic Biol Med* **11:** 81–128, 1991.
54. Mayanil CS, Kazmi SM and Baquer NZ, Na^+, K^+-ATPase and Mg^{2+}-ATPase activities in different regions of rat brain during alloxan diabetes. *J Neurochem* **39:** 903–908, 1982.
55. Gerbi A, Maixent JM, Barbey O, Jamme I, Pierlovisi M, Coste T, Pieroni G, Nouvelot A, Vague P and Raccah D, Alterations of Na^+, K^+-ATPase isoenzymes in the rat diabetic neuropathy: Protective effect of dietary supplementation with n-3 fatty acids. *J Neurochem* **71:** 732–740, 1998.
56. Greene DA, Lattimer SA, Carroll PB, Fernstrom JD and Finegold DN, A defect in sodium-dependent amino acid uptake in diabetic rabbit peripheral nerve. Correction by an aldose reductase inhibitor or *myo*-inositol administration. *J Clin Invest* **85:** 1657–1665, 1990.
57. Keller JN, Mark RJ, Bruce AJ, Blanc E, Rothstein JD, Uchida K, Waeg G and Mattson MP, 4-Hydroxynonenal, an aldehydic product of membrane lipid peroxidation, impairs glutamate transport and mitochondrial function in synaptosomes. *Neuroscience* **80:** 685–696, 1997.