

Dehydroepiandrosterone Prevents Oxidative Injury Induced by Transient Ischemia/Reperfusion in the Brain of Diabetic Rats

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Both chronic hyperglycemia and ischemia/reperfusion (IR) cause an imbalance in the oxidative state of tissues. Normoglycemic and streptozotocin (STZ)-diabetic rats were subjected to bilateral carotid artery occlusion for 30 min followed by reperfusion for 60 min. Rats had either been treated with dehydroepiandrosterone (DHEA) for 7, 14, or 21 days (2 or 4 mg/day per rat) or left untreated. Oxidative state, antioxidant balance, and membrane integrity were evaluated in isolated synaptosomes. IR increased the levels of reactive species and worsened the synaptic function, affecting membrane Na/K-ATPase activity and lactate dehydrogenase release in all rats. The oxidative imbalance was much severer when transient IR was induced in STZ-diabetic rats. DHEA treatment restored H₂O₂, hydroxyl radical, and reactive oxygen species to close to control levels in normoglycemic rats and significantly reduced the level of all reactive species in STZ-diabetic rats. Moreover, DHEA treatment counteracted the detrimental effect of IR on membrane integrity and function: the increase of lactate dehydrogenase release and the drop in Na/K-ATPase activity were significantly prevented in both normoglycemic and STZ-diabetic rats. The results confirm that DHEA, an adrenal steroid that is synthesized de novo by brain neurons and astrocytes, possesses a multitargeted antioxidant effect. They also show that DHEA treatment is effective in preventing both derangement of the oxidative state and neuronal damage induced by IR in experimental diabetes. *Diabetes* 49:1924–1931, 2000

Oxygen free radicals contribute to brain injury during cerebral ischemia (1,2). Because of high levels of polyunsaturated lipids, nervous tissue is markedly sensitive to oxygen free radical damage. Free radicals attack lipid membranes, and peroxidative propagation with neurodegeneration occurs by the consecutive production of other oxygen radical species. Several experimental studies indicate that this cascade of reactions induced by ischemia followed by recirculation causes membrane disintegration and irreversible energy failure, leading to the aggravation of brain edema and loss of neuronal functions (3,4).

There is now substantial epidemiological evidence that diabetes is a risk factor for cerebrovascular disease (5). Diabetic patients have at least twice the risk of stroke, from which they have a higher mortality and slower recovery (6). Besides the clustering of established risk factors that facilitate macrovascular and microvascular lesions, other mechanisms can play a role in the aggravated ischemic brain damage observed in diabetes. Studies show that both acute (7) and chronic (8) hyperglycemia modify the oxidative state of nervous tissue. Enhanced oxidative stress and changes in antioxidant balance, which lead to the release of excitatory neurotransmitters and disruption of ionic homeostasis, have been observed in both experimental and clinical diabetes (9). Thus, oxygen free radical generation appears to be enhanced by both cerebral ischemia/reperfusion and hyperglycemia, and it is thought to be among the primers of biochemical derangement observed in diabetic patients with cerebrovascular disease (10). Treatment with antioxidants reduces diabetic complications (11,12). Antioxidants normalize the endothelial function, exert a beneficial effect on platelet hyperaggregability, and decrease the occurrence of malformations in the offspring of diabetic rats (13). Promising strategies for prevention and treatment of diabetes complications using antioxidant compounds such as vitamin E and glutathione (GSH) have been proposed (14).

Dehydroepiandrosterone (DHEA) and dehydroepiandrosterone sulfate (DHEA-S), the most abundant adrenal steroids in the blood, are present in all regions of the human brain at concentrations above those in the plasma (15) and are synthesized de novo by brain neurons and astrocytes (16). Several roles, including the increase of neuronal and glial survival and differentiation, have been attributed to these neuroactive steroids (17,18), but the mechanism(s) responsible for these

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DCF, highly fluorescent 2',7'-dichlorofluorescein; DCFH, nonfluorescent 2',7'-dichlorofluorescein; DCFH-DA, 2',7'-dichlorofluorescein diacetate; DHEA, dehydroepiandrosterone; DHEA-S, dehydroepiandrosterone sulfate; GSH, glutathione; IR, ischemia/reperfusion; LDH, lactate dehydrogenase; NMDA, N-methyl-D-aspartic acid; OH[•], hydroxyl radical; ROS, reactive oxygen species; STZ, streptozotocin.

effects is not yet known. Although DHEA at very high pharmacological doses has been found to induce oxidative stress in rats by acting as a peroxisome proliferator (19), evidence indicates that at concentrations slightly above those found in human tissues, DHEA possesses a multitargeted antioxidant effect (20–23) and prevents tissue damage induced by acute (7) and chronic hyperglycemia (24,25). Moreover, we recently showed that DHEA treatment restores the redox state in synaptosomes of diabetic rats (26).

The present work examines the effect of chronic DHEA treatment on the oxidative brain damage produced by two combined conditions, chronic hyperglycemia and ischemia/reperfusion (IR), in isolated synaptosomes from streptozotocin (STZ)-treated rats. Bilateral carotid clamping with subsequent declamping in rats has been validated as a suitable experimental model mimicking human stroke, which produces both biochemical nervous tissue alteration and oxygen radical generation (27,28). Synaptosomes, the isolated terminal portions of axons that behave as metabolically autonomous minicells, provide a good experimental model to evaluate nervous degenerative processes and peroxidative events in cerebral cells (29). The results show that DHEA treatment ameliorates several markers of neuronal damage induced by free radicals caused by IR in diabetic rats.

RESEARCH DESIGN AND METHODS

Materials. ATP, dehydroisoandrosterone (5-androsten-3 β -ol-17-one, dehydro-epiandrosterone), STZ, (+)- α -tocopherol, NADH, reduced GSH, 2',7'-dichlorofluorescein diacetate, mono- and polyunsaturated fatty acid methyl-ester standards, and the internal standard heptadecanoic acid (17:0) were from Sigma Chemical (St. Louis, MO). Folin-Ciocalteu's phenol reagent and EDTA were from Merck (Darmstadt, Germany).

Treatment of animals. Male Wistar rats (Harlan-Italy, UD) weighing 250–280 g were cared for in compliance with the Italian Ministry of Health Guidelines (no. 86/609/CEE) and with the *Principles of Laboratory Animal Care* (NIH no. 85–23, revised 1985). They were provided with Piccioni pellet diet (no. 48, Gessate Milanese, Italy) and water ad libitum. Hyperglycemia was induced through a single injection of STZ (50 mg/kg) in the tail vein. Three days later, glycemia was measured (Sigma-kit, catalog no. 635) on blood collected from the heart (1 ml). Only rats with blood glucose levels above 20 mmol/l entered the experimental protocols. On the fourth day postinjection, hyperglycemic and control rats began oral DHEA treatment.

DHEA was given for 7, 14, or 21 days at 2 or 4 mg/day per rat. Crystalline DHEA was dissolved in 1 vol of 95% ethanol, mixed with 9 vol mineral oil, and given daily by gastric intubation. Controls received only the vehicle. At the end of the treatment period, normal rats and hyperglycemic rats, with or without DHEA, were submitted to transient IR and killed by aortic exsanguination. Transient cerebral ischemia was performed in anesthetized animals. Anesthesia was induced with a mixture of Ketavet 100 (Farmaceutici Gellini, Italy) and Rompum (xilazina; Bayer AG, Leverkusen, Germany) (4:1, vol:vol) by an intraperitoneal injection (0.5 ml mixture/200 g body weight). The rectal temperature was maintained close to 37.5–37.8°C by a heating lamp. Atropine sulfate (0.1 mg) and heparin (100 U) were injected intraperitoneally to prevent vago-reflex and coagulation. Ischemia was achieved by clamping the bilateral common carotid arteries with Scoville microclips for 30 min. Recirculation of blood flow was established by releasing the clips. Reperfusion was allowed for 60 min. At the end of this time, the rats were killed by decapitation after aortic exsanguination. Blood was collected and the plasma isolated. Cerebral hemispheres were chiseled and rapidly weighed and homogenized with Potter Elvehjem in 0.3 mol/l sucrose. Glycemia was evaluated with *o*-toluidina reagent (Sigma Kit, catalog no. 635).

Isolation of synaptosomal fraction. The tissue was homogenized in 0.3 mol/l sucrose, then centrifuged by sequential centrifugation. The method used for isolation has been described by Hajos (30). The final synaptosomal pellet from one rat was taken up in isolation medium to a final volume of 4.0–4.5 ml. The protein content was evaluated by Lowry et al.'s method (31) and was about 5 mg protein/ml.

Biochemical analysis on synaptosomal fractions. The oxidative state was determined by monitoring the generation of hydrogen peroxide (H₂O₂) by adding peroxidase from horseradish and acetylated ferrocytochrome c (32) to synaptosomal fractions. H₂O₂ release was evaluated as the increase of the

acetylated ferrocytochrome c oxidation rate and was monitored at 550 nm minus 540 nm using an absorption coefficient of 19.9 mmol · l⁻¹ · cm⁻¹ as described by Zoccarato et al. (33).

Hydroxyl radical (OH[•]) was quantified as described by Halliwell (34). Hydroxyl radicals attack the sugar deoxyribose and degrade it into fragments, some of which react with thiobarbituric acid, coloring the solution pink. The deoxyribose assay gives an approximate indication of the ability of the compound to interfere with iron ion-dependent site-specific Fenton chemistry.

Reactive oxygen species (ROS) were measured using probe 2',7'-dichlorofluorescein diacetate (DCFH-DA). DCFH-DA is a stable, nonfluorescent molecule that readily crosses the cell membrane and is hydrolyzed by intracellular esterases to nonfluorescent 2',7'-dichlorofluorescein (DCFH), which is rapidly oxidized in the presence of peroxides to highly fluorescent 2',7'-dichlorofluorescein (DCF). DCF is measured fluorimetrically (35).

Antioxidant levels were evaluated in terms of reduced GSH content by the Ellman method (36) and of α -tocopherol content as described by Burton et al. (37). After extraction of the sample (0.5 ml) with 1 ml *N*-heptane and brief centrifugation, the heptane phase was collected for high-performance liquid chromatography analysis. A Supercosil-plc-si column (25 cm × 4.6 mm; Supelco, PA) was used, the mobile phase being *N*-hexane-isopropanol (99:1, vol:vol) and the flow rate 2.0 ml/min; the fluorescence detector was set to 298 nm excitation and 325 nm emission.

Content of fluorescent chromolipids (4-hydroxynonenal plus malondialdehyde adducts) was measured as described by Esterbauer et al. (38). Total lipids were extracted as described by Folch et al. (39); the fluorescence intensity was evaluated at 360 nm excitation and 430 nm emission.

Integrity of synaptosomal membranes was evaluated in terms of lactate dehydrogenase (LDH; EC 1.1.1.27) release—determined spectrophotometrically at 340 nm by Lai and Clark's method (40)—and of Na/K-ATPase (EC 3.6.1.3) activity, assayed in a medium containing imidazole-HCl buffer pH 7.4, 120 mmol/l NaCl, 10 mmol/l KCl, 5 mmol/l MgCl₂, and 4 mmol/l ATP. The reaction was started by adding 25 μ g of synaptosomal membrane protein and carried out as described by Shallom and Katayare (41).

Fatty acid content of synaptosomal membrane was determined by gas-liquid chromatography. Synaptosomal lipids were extracted by Folch et al.'s method (39), methylated with 5 ml NaOH in MetOH 0.5 N and hydrolyzed with 5 ml HCl 6 N (42). As internal standard, heptadecanoic acid (17:0) was added to the solution. The mixture was vortexed and centrifuged. The CHCl₃ phase was then removed and evaporated under vacuum. The samples were redissolved in 300 μ l CHCl₃. Gas-liquid chromatography was performed by injecting 3 μ l of sample into a Perkin Elmer-Sigma 300 Dual Chromatograph equipped with flame ionization 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 was 200°C for 30 min, increased linearly to 220°C at 5°C/min, then held 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).

Histopathological evaluation. A neuropathological study was performed in six rats (two normoglycemic, two STZ-treated, and two STZ-treated plus DHEA 4 mg/day for 21 days) subjected to 30 min of ischemia and a subsequent 60 min of reperfusion. Animals were perfused via the ascending aorta with 4% phosphate-buffered formaldehyde (pH 7.35), and then the brains were cut coronally in 3-mm thick slices, dehydrated, and paraffin embedded. Coronal sections of 5 μ m were stained with cresyl violet and acid fuchsin, and the ischemic neuronal damage was assessed in the hippocampus, the cingulate, and the parietal cortex. In consideration of the findings of Li et al. (43), three types of neuropathological changes were considered: preacidophilic neurons, acidophilic neurons, and sponginess.

Statistical analysis. All results are presented as means \pm SD. Differences between means were analyzed for significance using the one-way analysis of variance test with the Bonferroni post test (44).

RESULTS

STZ induced a marked increase in the glucose level 3 days after STZ injection (data not shown). Hyperglycemia was reconfirmed at the end of the experiments in STZ-treated rats (glucose level ~30 mmol/l) and was not modified by DHEA treatment throughout the experiment. DHEA levels in plasma and in brain synaptosomes are reported in Table 1. During DHEA treatment, the plasma level of DHEA reached values slightly above those found in normal humans, whereas synaptosomal levels of DHEA were unchanged.

TABLE 1

Level of DHEA in plasma and brain synaptosomes isolated from normal or diabetic rats exposed to IR, with or without DHEA treatment

Group	Plasma (ng/ml)	Synaptosomes (ng/mg protein)
Control	1.69 ± 1.16	0.41 ± 0.23
IR	8.20 ± 3.97	0.31 ± 0.14
DHEA + IR	12.70 ± 6.95*	0.61 ± 0.26
STZ + IR	6.05 ± 3.44	0.40 ± 0.14
STZ + DHEA + IR	11.40 ± 5.60*	0.66 ± 0.31

Data are means ± SD of 7–10 rats per group. DHEA treatment was 4 mg/day for 21 days. * $P < 0.01$ vs. control.

Oxidative state. H_2O_2 levels of synaptosomal fraction isolated from diabetic rats treated with DHEA 2 or 4 mg/day for 7, 14, or 21 days and subjected to IR are reported in Fig. 1A. The protective effect of DHEA on H_2O_2 generation induced by IR became discernible after feeding with 4 mg/day of steroid for 14 days ($P < 0.05$). DHEA 2 mg/day is effective only after 21 days ($P < 0.05$).

In synaptosomal fractions isolated from rats treated with 4 mg/day of DHEA for 21 days, all parameters of the oxidative state were measured (Table 2). We previously showed that DHEA alone had no effect on synaptosomal level of ROS in normoglycemic rats (26). Here we show that H_2O_2 , OH^\bullet , and ROS levels were markedly higher in rats subjected to IR than in controls. In rats subjected to the combined conditions (IR and hyperglycemia), all parameters were much more markedly raised and were significantly different from both

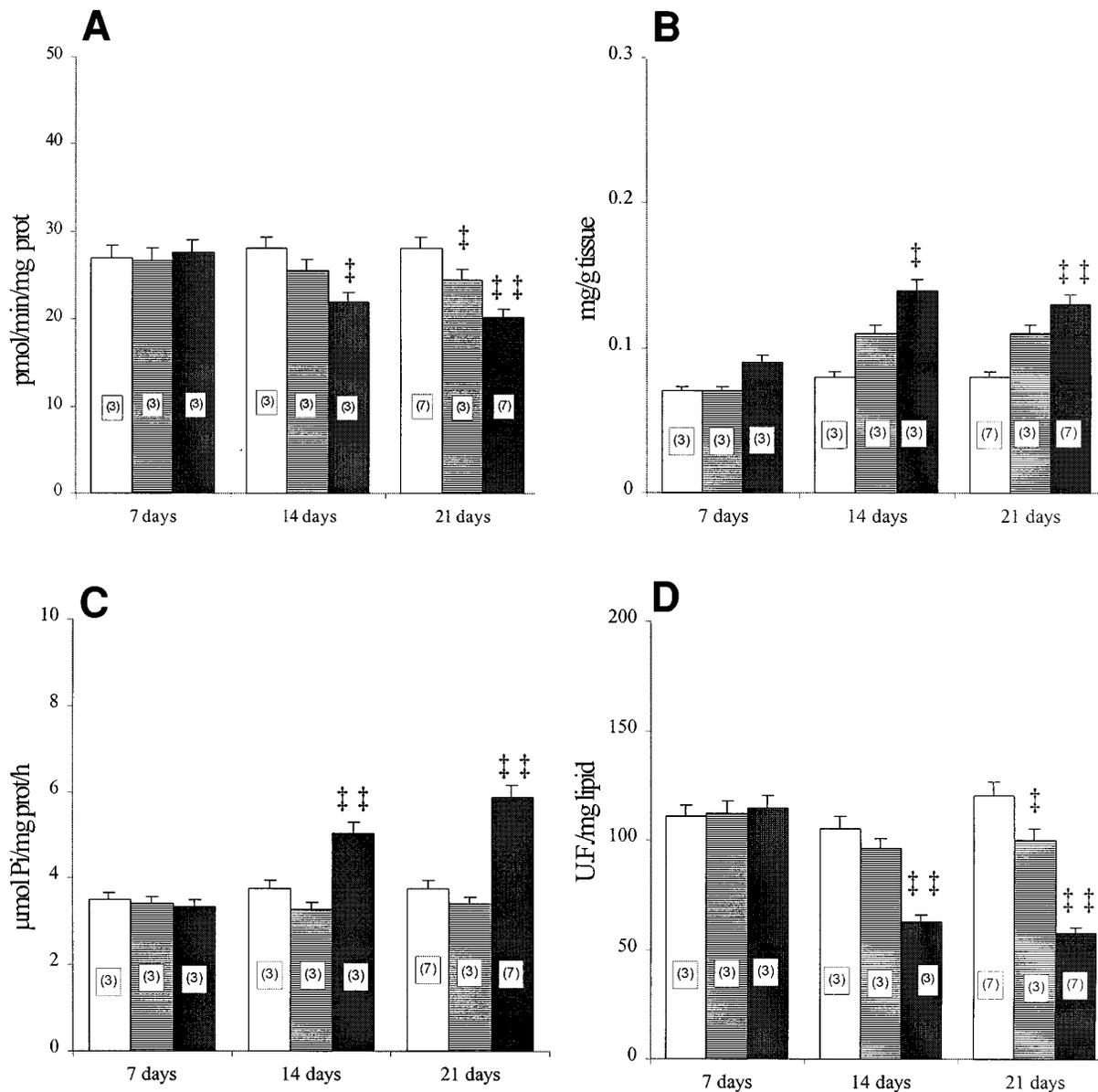


FIG. 1. H_2O_2 (A), total GSH (B), Na/K-ATPase (C), and fluorescent chromolipids (D) in brain synaptosomes isolated from diabetic rats exposed to IR with or without DHEA treatment for 7, 14, or 21 days. Data are means ± SD. Number of animals treated is in brackets. † $P < 0.05$ vs. STZ + IR; ‡ $P < 0.01$ vs. STZ + IR. □, STZ + IR; ▨, STZ + IR + DHEA 2 mg; ■, STZ + IR + DHEA 4 mg.

TABLE 2
H₂O₂, OH[°], and ROS in brain synaptosomes isolated from normal or diabetic rats exposed to IR with or without DHEA treatment

Group	H ₂ O ₂ (pmol · min ⁻¹ · mg protein ⁻¹)	OH [°] (ABS 523 nm)	ROS (UF/mg protein)
Control	17.92 ± 1.62	1.03 ± 0.05	0.96 ± 0.12
IR	21.91 ± 0.88*	1.25 ± 0.05*	1.74 ± 0.11*
DHEA + IR	19.72 ± 0.69†	1.07 ± 0.03†	1.03 ± 0.13†
STZ + IR	27.97 ± 2.49*†	1.48 ± 0.06*†	2.10 ± 0.32*†
STZ + DHEA + IR	20.17 ± 1.68‡	1.17 ± 0.20‡	1.06 ± 0.06‡

Data are means ± SD of 7–10 rats per group. DHEA treatment was 4 mg/day for 21 days. **P* < 0.05 vs. control; †*P* < 0.05 vs. IR; ‡*P* < 0.05 vs. STZ + IR. ABS, absorbance; UF, units of fluorescence.

control and IR-treated rats. DHEA treatment in normoglycemic rats exposed to IR restored the levels of all reactive species to close to control levels. Also in hyperglycemic rats, DHEA treatment strongly reduced H₂O₂, OH[°], and ROS levels. Thus, DHEA treatment is very efficient at preventing derangement of the oxidative state induced by the two combined oxidative stimuli.

Antioxidant barriers. The GSH level, which is reduced after 30 min of ischemia (data not shown), was rapidly restored during reperfusion in normoglycemic rats. It was markedly decreased in diabetic rats exposed to IR (Fig. 1B and Table 3). DHEA (4 mg/day for 21 days) partially prevented the loss of tripeptide in the latter group. A marked depletion of α-tocopherol (by 50%) occurred during IR in both normoglycemic and hyperglycemic rats. DHEA completely prevented the reduction of α-tocopherol in normoglycemic rats that underwent IR, and it only partially prevented the loss in hyperglycemic rats (Table 3).

Integrity of membrane. The dose of 4 mg/day DHEA (for 21 days) partially reduced both the increased release of LDH and the drop of Na/K-ATPase activity observed in IR-exposed rats (Table 3), whereas 2 mg/day DHEA was ineffectual (Fig. 1C). In diabetic rats exposed to IR, the release of LDH doubled and Na/K-ATPase activity dropped much more markedly than in normoglycemic IR rats. In diabetic rats exposed to IR, DHEA showed a partially preventive effect on both the release of LDH and the activity of ATPase, restoring them to values found in normal rats exposed to IR.

Lipid peroxidation. Isolated synaptosomes obtained from normoglycemic rats exposed to IR showed a significant increase of fluorescent adducts compared with controls, an increase that was partially prevented by DHEA (Fig. 2). Hyperglycemia dramatically increased the level of fluorescent

adducts. DHEA prevented the increase of fluorescent chromolipid adducts, restoring the level to that found in normoglycemic rats subjected to IR (Figs. 1D and 2).

Membrane fatty acids. The content of some unsaturated fatty acids of the synaptosomal membrane was determined. The content of oleic, arachidonic, and docosahexanoic acids in normoglycemic and hyperglycemic IR rats was reduced by 30%. DHEA treatment (4 mg/day for 21 days) restored them to control values in normoglycemic rats, but it only partially prevented this imbalance in diabetic rats (Table 4).

Neuropathological changes. Severe neuronal damage was evident in CA4 cells (hilus of dentate gyrus) of the hippocampus in hyperglycemic rats subjected to IR (Fig. 3); preacidophilic and acidophilic neurons were bilaterally present and numerous (>20%) in both animals. A similar pattern of damage was observed in the neurons of layers III–V of the cingulate cortex and, though not in all cases, of the parietal cortex. Neuronal damage was also observed in DHEA-treated rats in the same areas; however, the number of ischemic neurons was lower (Fig. 3). In one of these animals, the number of damaged neurons in the CA4 sector was very low in one hemisphere and below 10% in the other.

DISCUSSION

Active oxygen species are involved in STZ-induced destruction of β-cells. However, STZ is eliminated from the body in a few hours (45); thus, any interference would be expected to occur in the pro-oxidant effect of hyperglycemia and IR.

DHEA treatment markedly reduces the oxidative imbalance induced by transient ischemia followed by reperfusion in the synaptosomes obtained from the brain of both normoglycemic and STZ-diabetic rats. Tissue energy failure and free radical production have been regarded as important mechanisms respon-

TABLE 3
Total GSH and α-tocopherol content, LDH release, and Na/K-ATPase activity in brain synaptosomes isolated from normal or diabetic rats exposed to IR with or without DHEA treatment

Group	GSH (mg/g tissue)	α-Tocopherol (pmol/mg protein)	LDH (% release)	Na/K-ATPase (μmol Pi · mg protein ⁻¹ · h ⁻¹)
Control	0.18 ± 0.02	498.74 ± 47.14	8.53 ± 2.13	7.50 ± 0.84
IR	0.16 ± 0.03	276.20 ± 70.54*	22.70 ± 2.74*	5.52 ± 0.61*
DHEA + IR	0.15 ± 0.01	488.40 ± 78.35†	16.41 ± 0.35*†	6.49 ± 0.33*†
STZ + IR	0.08 ± 0.01*†	238.32 ± 80.16*	41.82 ± 4.13*†	3.75 ± 0.34*†
STZ + DHEA + IR	0.13 ± 0.02*‡	355.42 ± 49.50*‡	23.80 ± 1.69*‡	5.85 ± 0.31*‡

Data are means ± SD of 7–10 rats per group. DHEA treatment was 4 mg/day for 21 days. **P* < 0.05 vs. control; †*P* < 0.05 vs. IR; ‡*P* < 0.05 vs. STZ + IR.

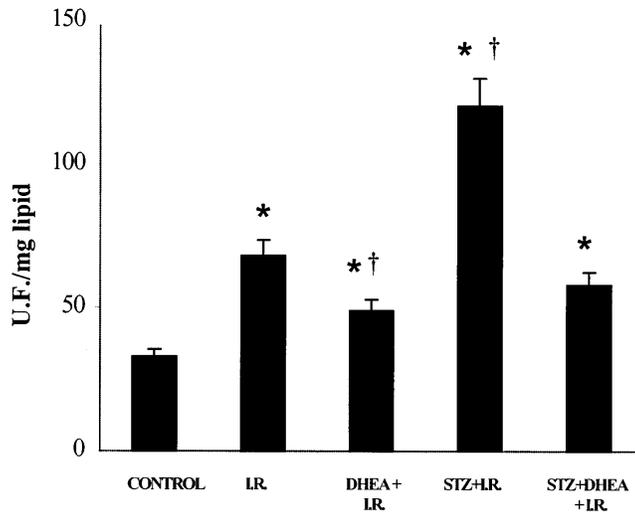


FIG. 2. Fluorescent chromolipids in brain synaptosomes isolated from normal or diabetic rats exposed to IR with or without DHEA treatment (4 mg/day for 21 days). Data are means of 7–10 rats \pm SD. * $P < 0.05$ vs. control; † $P < 0.05$ vs. IR.

sible for brain damage after ischemia. They lead to disruption of ionic homeostasis (46), increased concentration of Ca^{2+} (47), release of excitatory neurotransmitters (48), platelet-activating factor synthesis (49), and lipid-membrane degradation and cell death. The results of our experiments on normoglycemic rats confirm previous studies (50) showing that IR creates an imbalance in the oxidative status of nervous tissue and demonstrating that the presynaptic region of the neuron is damaged by oxygen reactive species. In our study, IR not only increased the levels of reactive species and reduced the content of the nonenzymatic antioxidants, but also worsened synaptic function affecting membrane Na/K-ATPase activity and damaging the synaptosomal membrane, as suggested by the increase of LDH release and changes of fatty acid content. The protective effect of DHEA is in keeping with a substantial, although not unique, role of free radical overproduction in ischemic insult: DHEA treatment restores all reactive species detected and endogenous nonenzymatic antioxidants to levels close to control values. Also, the detrimental effects of IR on membrane integrity and function are ameliorated by DHEA treatment: it slows the increase of LDH release to ~50% of that observed in normoglycemic rats under the same ischemic conditions and improves synaptosomal Na/K-ATPase activity.

The oxidative imbalance in brain synaptosomes is much severer when transient IR is induced in STZ-diabetic rats. The

effect of acute or chronic hyperglycemia on ischemic brain damage has, until recently, been a matter for debate. It has been reported that STZ-induced diabetes protects stroke-prone spontaneous hypertensive rats against stroke (51) and that hyperglycemia preserves the brain oxidative phosphorylation during ischemia better than normoglycemia does (49). The hypothesis has been put forward that hyperglycemia provides sufficient substrate to prolong cellular energy metabolism (52). However, despite the hypothetical beneficial effects on the energy supply, it is now generally accepted that hyperglycemia worsens the brain damage induced by IR (53). Autopsy studies in humans suggest that diabetic patients are more susceptible to lacunar infarction than are normoglycemic subjects (6). Moreover, experimental evidence exists that preinsult hyperglycemia causes an increase in brain damage resulting from transient ischemia (54). A direct correlation between superoxide radical generation and histopathological changes of pyramidal cells after cerebral IR has been reported (55), as has the exacerbation of neurological and histopathological outcomes after focal cerebral ischemia in Cu-Zn superoxide dismutase gene knockout mutant mice (56). Besides the other well-known mechanisms that increase free radical production in diabetes (57), the sharp drop in intracellular pH induced by hyperglycemic ischemia favors free radical production (58). Here we show that all parameters of both oxygen reactive species generation and synaptosomal membrane integrity and function are worsened in rats submitted to the chronic hyperglycemia and IR combined.

The importance of antioxidant treatment in preventing neuronal damage has recently been emphasized (23,59–61). Vitamin E (62) and a novel free radical scavenger (59) protect against changes in membrane permeability caused by peroxidation and attenuate ischemic and postischemic brain swelling. Here we show, for the first time, that an endogenous steroid, directly synthesized in the brain, clearly protects synapses against oxidative stress. The protective effect, already evident in synaptosomes of IR brain, became much more notable in diabetic rats subjected to IR. Hydroxyl levels, as well as the production of H_2O_2 and ROS, are markedly reduced by DHEA treatment, suggesting that DHEA might be an excellent scavenger in brain as well as in other tissues (26). However, although DHEA treatment increases plasma levels of the steroid, it does not modify synaptosomal DHEA levels. Thus, it is difficult to reconcile the lag time needed for DHEA to become active and the detection of unchanged synaptosomal levels of DHEA during the treatment in the present study with a direct interception of free radical species by the steroid. The possibility that inserting DHEA into the lipid membranes makes them more resistant to oxidative stress

TABLE 4

Fatty acid content of synaptosomal membrane isolated from normal or diabetic rats exposed to IR with or without DHEA treatment

Group	Oleic acid ($\mu\text{mol/g}$ brain)	Arachidonic acid ($\mu\text{mol/g}$ brain)	Docosahexanoic acid ($\mu\text{mol/g}$ brain)
Control	3.10 ± 0.22	0.96 ± 0.14	0.97 ± 0.14
IR	$2.05 \pm 0.23^*$	$0.64 \pm 0.08^*$	$0.56 \pm 0.14^*$
DHEA + IR	$2.92 \pm 0.48^\dagger$	$0.84 \pm 0.14^\dagger$	$0.80 \pm 0.10^\dagger$
STZ + IR	$2.06 \pm 0.18^*$	$0.62 \pm 0.04^*$	$0.65 \pm 0.08^*$
STZ + DHEA + IR	$2.47 \pm 0.08^{*\ddagger}$	$0.78 \pm 0.04^{*\ddagger}$	$0.87 \pm 0.08^\ddagger$

Data are means \pm SD of 7–10 rats per group. DHEA treatment was 4 mg/day for 21 days. * $P < 0.05$ vs. control; † $P < 0.05$ vs. IR; ‡ $P < 0.05$ vs. STZ + IR.

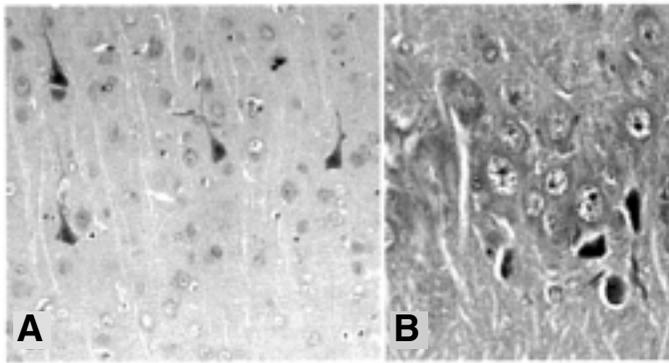


FIG. 3. Histopathological damage in hyperglycemic rats treated with DHEA (4 mg/day for 21 days) subjected to ischemia after 60 min of reperfusion. **A:** Damaged neurons in parietal cortex (200 \times). **B:** Ischemic neurons in C4 area (400 \times). Cresyl violet-acid fuchsin.

may be considered. The intercalation of DHEA into the lipid membranes has been suggested as the mechanism responsible for the change in shape induced in vitro by DHEA in human red blood cells (63). Very recently, it has been shown that mitochondrial superoxide production plays a key role in hyperglycemic damage of tissues (64), and DHEA has been reported to change the fatty acid composition of mitochondrial membrane phospholipids in rats (65). The possibility that in biological membranes DHEA becomes incorporated as fatty acid esters, which escape recognition by radioimmunoassay, might explain the similar levels of DHEA that we measured in synaptosomes of rats treated and untreated with the steroid. However, some indirect effects of DHEA should also be considered. In nervous tissue, ischemia induces the expression of the mRNA level of tumor necrosis factor- α (66) that activates the nuclear factor- κ B pathway (67) and produces ROS. DHEA selectively inhibits tumor necrosis factor- α production in astrocytes (68). It is, however, also possible to speculate on many other mechanisms, such as a change in the level of other unidentified substances that attenuate oxidative stress induced by hyperglycemia and IR.

The protective effect of DHEA is confirmed by the restoration of nonenzymatic barriers (GSH and α -tocopherol). The reduced oxidative imbalance leads to a reduced peroxidation of lipid membranes, as indicated by the reduction of chromolipid levels in the synaptosomes of DHEA-treated animals. 4-Hydroxynonenal, which conjugates to many different proteins, including Na/K-ATPase (69), is considered to play an early role in impairing the multiple membrane transport and signaling systems before synaptic degeneration after ischemia (70). Besides DHEA's effect via the reduction of lipid peroxide products, the decrease of polyol pathway activity may contribute to preserving Na/K-ATPase activity. In fact, our previous studies have demonstrated that DHEA treatment partially suppresses the increase of brain aldose reductase activity observed in diabetes (24).

The loss of Na/K ATPase activity produces membrane depolarization, opening N-methyl-D-aspartic acid (NMDA) receptor channels and favoring the massive Ca^{2+} influx (71) through channels gated by glutamate receptors (47). Recent studies show that DHEA, via mechanisms not yet clear, protects neurons against excitatory amino acid-induced neurotoxicity after cerebral ischemia (17) and modulates GABA-

gate chloride ion influx in synaptosomes (72). We suggest that the powerful ameliorative effect of DHEA on glutamate-induced neurotoxicity may be dependent on its antioxidant properties, which lead to the reduction of aldehyde products derived from lipid oxidation.

The serious derangement of synaptic membrane integrity and function induced by IR is confirmed by the increase of LDH release and by the changes of fatty acid membrane content. Both effects are counteracted by DHEA treatment; we found less derangement of polyunsaturated fatty acid content of membranes in DHEA-treated than in STZ-alone rats. The neuropathological alterations observed in this study reproduce the well-known distribution pattern of neuronal damage related to IR in hyperglycemic rats (10,43,73). In particular, damaged neurons were observed in the hippocampus and neocortex of all rats. Even though the neuronal damage appeared to be less severe in DHEA-treated rats, the small sample size suggests that this result cannot be considered conclusive.

Whatever the mechanism, the data clearly demonstrate that DHEA supplementation protects synaptosomes of STZ-diabetic rats against the additional oxidative damage induced by IR. The high concentration of NMDA and glutamate receptors and membrane transport and signaling systems make synapses particularly vulnerable to oxidative stress. Data are lacking as to whether, as at the adrenal level, DHEA biosynthesis in astrocytes and neurons decreases with advancing age; if that is the case, it may be argued that the maintenance of DHEA biosynthesis at the neuronal level influences the severity of brain damage induced by cerebral ischemia and diabetes. Moreover, because all pathological and neurosurgical situations in humans in which cerebral perfusion is temporarily reduced are characterized by enhanced radical generation, the antioxidant effect of DHEA points the way toward an additional therapeutic approach to neuronal failure in diabetic cerebrovascular disease.

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