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29 November 2024

Oxidative stress and kidney dysfunction due to ischemia/reperfusion in rat: Attenuation by dehydroepiandrosterone

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Oxidative stress and kidney dysfunction due to ischemia/reperfusion in rat: Attenuation by dehydroepiandrosterone.

Background. The pathogenesis of ischemia/reperfusion (I/R) involves generation of reactive oxygen and nitrogen species. This in vivo study investigates the effect of dehydroepiandrosterone (DHEA), a physiologic steroid with antioxidant properties, on oxidative balance and renal dysfunctions induced by monolateral I/R.

Methods. Normal and DHEA-treated rats (4 mg/day × 21 days, orally) were subjected to monolateral renal I/R (30 minutes/6 hours). The oxidative state was determined by measuring hydrogen peroxide level and activities of glutathione-peroxidase, catalase, and superoxide dismutase. Tumor necrosis factor-α (TNF-α) and nitric oxide production and inducible nitric oxide synthase (iNOS) levels were also measured. Hydroxynonenal content was used to probe lipid peroxidation. Functional parameters determined were creatinine levels and Na/K-ATPase activity. Immunohistochemical and morphologic studies were also performed.

Results. A markedly pro-oxidant state was evident in the kidney of rats subjected to I/R. Both hydrogen peroxide and reactive nitrogen species (nitric oxide and iNOS) increased, whereas antioxidants decreased. Oxidant species induce TNF-α increase, which, in turn, produces lipoperoxidative processes, as documented by the increased hydroxynonenal (HNE) level. As final result, impaired renal functionality, hydropic degeneration, and vacuolization of proximal convolute tubules were observed in kidneys of I/R rats. DHEA pretreatment improved the parameters considered.

Conclusion. I/R induces oxidative stress and consequently damages the proximal convolute renal tubules. Rats supplemented with DHEA and subjected to I/R had reduced pro-oxidant state, oxidative damage, and improved renal functionality, indicating an attenuation of oxidative injury and dysfunctions mediated by I/R.

Ischemia/reperfusion (I/R) is an important cause of organ dysfunction often leading to acute kidney failure, causing high mortality among patients in intensive care who require dialysis [1]. The severity of the injury depends on the duration of ischemia and subsequent reperfusion. Reperfusion, although essential for the survival of ischemic renal tissue, causes additional damage. Oxidative stress is involved in kidney injury induced by I/R [2, 3]. Increased reactive oxygen species (ROS) generation occurs after reperfusion, leading to oxidative damage and dysfunction [4, 5]. Different experimental models have shown that antioxidant therapy can protect against oxidative damage induced by I/R [6, 7]. This protective effect may be related to the ability of antioxidant compounds to normalize early intracellular events linked to the progression of oxidative damage [8, 9].

A number of mechanisms explain tissue I/R injury. In addition to reduced glomerular filtration and accumulation of leukocytes [10], ROS, reactive nitrogen species (RNS) generation, and the loss of antioxidant defense are also considered to play key roles. Many of these reactive molecules activate the signaling mechanisms that culminate in tumor necrosis factor (TNF) production [11]. TNF-α is a proinflammatory cytokine capable of upregulating its own expression, as well as the expression of other genes important in the inflammatory response [12]. TNF-α, lipopolysaccharides, and I/R increase inducible nitric oxide synthase (iNOS) activity to synthetize nitric oxide [13, 14]. Nitric oxide production may play several roles in renal pathophysiology, including induction of tubular damage. Prevention or reduction of nitric oxide generation reduces nitric oxide renal injury [15], and the increased generation of nitric oxide is capable of inducing intracellular oxidizing reaction and cell death [16]. Thus, correction of the oxidant/antioxidant imbalance in I/R rats is an important approach to reducing the risk of developing acute kidney failure.

This study evaluates the effect of treatment with dehydroepiandrosterone (DHEA) on oxidative damage and renal dysfunction in a model of monolateral renal I/R. DHEA, a multifunctional steroid secreted by the adrenal gland and brain [17], can protect many tissues against oxidative damage [18–20]. Its physiologic role is unclear, although studies show a beneficial effect on vascular function, atherosclerosis, and complications of diabetes mellitus [21–23]. Here, we show that DHEA treatment interferes with the early pro-oxidant responses and with the TNF-α increase caused by I/R, reducing oxidative damage and ameliorating the functionality of the proximal tubules, the portion most susceptible to injury caused by I/R [24].

METHODS

Antibodies used in immunoblotting assay were all from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Unless specifically indicated, all reagents were from Sigma Chemical Co. (St. Louis, MO, USA).

Treatment of animals

Male Wistar rats (Harlan-Italy, Udine, Italy) weighing 220 to 250 g were cared for in compliance with the Italian Ministry of Health Guidelines (no. 86/609/EEC) and with the Principles of Laboratory Animal Care (NIH no. 85-23, revised 1985). They were provided with a pellet diet (Piccioni no. 48, Gessate Milanese, Italy) and water ad libitum.

The rats were randomly divided into five groups (*N* = 8 to 10): (*1*) control, (*2*) sham-operated, (*3*) DHEA alone, (*4*) I/R (30 minutes/6 hours), and (*5*) DHEA plus I/R. DHEA was given for 21 days at 4 mg/day per rat before I/R. Crystalline DHEA was dissolved in 1 volume of 95% ethanol, mixed with 9 volumes mineral oil and given daily by gastric intubation. Controls received vehicle alone. At the end of the treatment period, rats with or without DHEA pretreatment were anesthetized and submitted to transient monolateral renal ischemia. Anesthesia was induced with a mixture of Ketavet 100 (Farmaceutici Gellini, Ltd., Aprilia, Italy) and Rompum (xilazina; Bayer AG, Leverkusen, Germany) (4:1, vol/vol) by intraperitoneal injection (0.5 mL of mixture/200 g body weight). Body temperature was maintained close to 37.5°C to 37.8°C with a heating lamp. An abdominal incision was made and perirenal fat, which supplies small arteries to the surface of the kidney, was stripped off. The vascular pedicle of the left kidney was then occluded with a nontraumatic vascular clamp for 30 minutes. At the end of this ischemic period, the right kidney was removed and recirculation of blood flow was established in the left kidney by releasing the clamp. Reperfusion was continued for 6 hours, after which the rats were killed by aortic exsanguination. Sham-operated rats were subjected to identical surgical procedures except for renal I/R. Blood was collected and the serum isolated. Ischemic kidney was removed and weighed immediately. Two coronal sections of approximatively 0.4 cm thickness were cut from the middle portion of the kidney. One slice was placed in 4% formaldehyde solution in 50 mmol/L phosphate buffer, pH 7.4, for morphologic studies; the second was immediately frozen under liquid nitrogen and stored at −80°C for immunohistochemical studies. The rest of the kidney was homogenized in a Potter Elvejhem device with different procedures.

Isolation of subcellular fractions

An aliquot (1 mL) of kidney homogenate (20%, wt/vol) prepared in 1.15% KCl was centrifuged at 15,000*g* for 18 minutes at 4°C; the entire supernatant obtained was centrifuged at 100,000*g* for 40 minutes at 4°C to obtain the cytosolic fractions [23]. A second portion of the kidney was homogenized (20%, wt/vol) in 0.25 mol/L sucrose, 5 mmol/L Tris-HCl, pH 7.4, medium to prepare the crude kidney particulate fraction. It was centrifuged at 1000*g* for 10 minutes at 4°C to remove nuclei and intact cells, the supernatant fraction (I) being retained; the pelleted nuclei and intact cells were rehomogenized with 1 mL of the same medium and centrifuged under the same conditions to obtain a second supernatant (II). The two supernatant fractions (I + II) were pooled and centrifuged at 40,000*g* for 30 minutes at 4°C. The final pellets were resuspended in fresh homogenising medium to a final concentration of 1 g equivalent of tissue/milliliter and stored at −80°C (crude particulate fraction) [25].

Pro-oxidant state

The pro-oxidant state was determined by monitoring generation of hydrogen peroxide (H_2O_2), adding the followings in cuvette (1 mL final volume): 7.4 IU horseradish peroxidase, 40 μmol/L acetylated ferrocytochrome-c, 5 μmol/L p-hydroxyphenilacetic acid, and 100 μL cytosolic fractions. H_2O_2 release was evaluated as the increase of acetylated ferrocytochrome-c oxidation rate, and 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 [26]. The nitrite/nitrate concentration in serum was used as an indicator of nitric oxide synthesis. Nitrates in serum samples were stoichiometrically reduced to nitrites by incubation of 250 μL of sample for 15 minutes at 37°C, in the presence of 1 IU/mL nitrate reductase, 500 μmol/L nicotinamide adenine dinucleotide phosphate (reduced form) (NADPH), and 50 μmol/L flavin adenine dinucleotide (FAD) in a final volume of 400 μL. When nitrate reduction was complete, unused NADPH, which interferes with subsequent nitrite determination, was oxidized by 100 IU/mL lactate dehydrogenase and 100 mmol/L sodium pyruvate in a final reaction volume of 500 μL and incubated for 5 minutes at 37°C [27]. Subsequently,

total nitrites in the serum were assayed by adding 500 μL of Griess reagent (4% sulphanilamide and 0.2% naphtylen-diamide in 10% phosphoric acid) to each sample [28].

Western blot analyses

TNF-α and iNOS were detected on cytosolic extracts using the method of Laemmli [29]. Aliquots of cytosol containing ≅ 60 μg proteins were resolved on 7.5% and on 12% sodium dodecyl sulfate (SDS)-polyacrylamide gels for TNF-α and iNOS, respectively, then blotted onto nitrocellulose membranes (Amersham Italy; Cologno Monzese, Milano, Italy). The membranes were blocked with 5% (wt/vol) nonfat dry milk in 5 mmol/L Tris-HCl, pH 7.4, containing 200 mmol/L NaCl and 0.05% (vol/vol) Tween 20 [Tris-buffered saline (TBS)-Tween] for 1 hour at 25°C, then incubated with goat polyclonal antibodies against TNF-α diluted 1:100 or rabbit polyclonal antibodies against iNOS diluted 1:200. The membranes were incubated with appropriate peroxidase-labeled secondary antibody prepared in TBS-Tween containing 2% (wt/vol) nonfat dry milk. Immunoreactive proteins were detected with the chemiluminescence assay (ECL) (Amersham) and subsequent exposure to film for 2 to 10 minutes. Anti-β-actin antibodies served as loading control for the cytosolic extract.

Antioxidant enzyme activity in cytosol

Glutathione-peroxidase Se-dependent (GSH-PX) activity was assayed by the method described by Flohè and Gunzler [30]. A mixture (final volume of 1 mL) containing 0.6 mol/L EDTA diluted in 6 mmol/L potassium phosphate buffer, pH 7.0, 100 μL of cytosol (diluted 1:100), 1.37 IU GSH-reductase, 1 mmol/L GSH, and 0.1 mmol/L sodium-azide was incubated for 10 minutes at 37°C. After incubation, 0.15 mmol/L NADPH diluted in 0.1% (wt/vol) NaHCO$_3$ was added to the mixture; 0.15 mmol/L H$_2$O$_2$, used as substrate, was then added to start the reaction. A kinetic analysis at 340 nm was performed and monitored for three minutes. Catalase activity was evaluated following the method of Aebi [31]. A mixture (final volume 3 mL) was directly prepared in cuvette: 1 mL of 30 mmol/L H$_2$O$_2$ diluted in 50 mol/L sodium phosphate buffer, pH 7.0, and 2 mL of cytosol (diluted 1:100) were monitored at 240 nm for 1 minute. A blank was prepared for each sample. Cu/Zn superoxide dismutase (SOD) activity was assayed as described by Flohè and Otting [32]. A solution (100 mL) was prepared with 5 mmol of xanthine diluted in 0.001 N sodium hydroxide, 2 mmol of cytochrome C, and 50 mmol/L phosphate buffer, pH 7.8, containing 0.1 mmol/L EDTA; 50 μL of cytosol were added to 2.9 mL of the solution. To start the reaction, 2.5 IU xanthine oxidase were added to the prepared mixture and a kinetic analysis was performed at 550 nm for 3 minutes.

End products of lipid peroxidation

Hydroxynonenal (HNE) concentration was also determined on fresh cytosolic fractions by the method of Esterbauer et al [33]. An aliquot of cytosol (200 μL) was extracted in an equal volume of a solution of acetic acid:acetonitrile (4:96, vol:vol). After centrifugation at 250*g* for 20 minutes at 4°C, 50 μL of supernatant were injected into a high-performance liquid chromatography (HPLC) Symmetry C$_{18}$ column (5 mm, 3.9 × 150 mm). The mobile phase used was acetonitrile:bidistilled water (42%,vol:vol). The HNE concentration was calculated by comparison with a standard solution of HNE (Calbiochem-Novabiochem Corp., La Jolla, CA, USA) of known concentration.

Functional parameters

Creatinine levels were determined on serum samples taken 6 hours after reperfusion from the abdominal aorta artery, using a commercially available kit (Sigma Chemical Co.). The results are expressed as milligrams per deciliter.

On crude kidney particulate fraction, Na/K-ATPase activity was assayed in a medium containing imidazolom-HCl buffer, pH 7.4, 120 mmol/L NaCl, 10 mmol/L KCl, 5 mmol/L MgCl$_2$, and 4 mmol/L adenosine triphosphate (ATP). The reaction was started by adding 25 μg of crude particulate protein, and carried out as described by Shallom and Katayare [34].

Morphologic and immunohistochemical studies

At the end of the reperfusion phase, a coronal section of the kidney was fixed overnight at 4°C in buffered 4% formaldehyde solution, pH 7.4. Paraffin-embedded sections were prepared and stained with the hematoxylin-eosin technique to evaluate damage of the proximal convolute tubules. Morphologic changes due to I/R injury were evaluated on 20 proximal tubules from the superficial and middle cortex using the following scoring system: 0 = normal histology; 1 = slight alteration (loss of brush border, mild hydropic degeneration); 2 = mild (intensive hydropic degeneration, mild vacuolization); 3 = moderate (shrunken nuclei, intensive vacuolization); 4 = severe (necrotic /apoptotic cells, denudation/rupture of basement membranes); and 5 = necrosis (total necrosis of the tubule).

Paraffin-embedded sections were prepared from frozen slices of kidney. The sections were deparaffinized, rehydrated, then immunostained to evaluate the presence of HNE-protein adducts. For this purpose, sections were incubated with an anti-HNE hystidine polyclonal antibody (Alpha Diagnostic International, San Antonio, TX, USA) in phosphate-buffered saline (PBS), pH 7.4, containing 1% Tween 20 (vol/vol) and 1% bovine serum albumin (wt/vol) as described elsewhere [35]. Peroxidase-
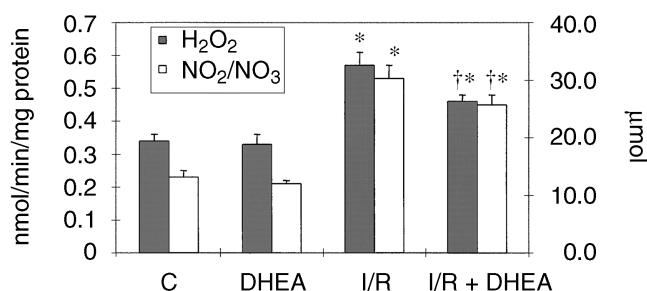
Fig. 1. Level of hydrogen peroxide (H_2O_2) in kidney cytosolic fraction and concentration of NO_2/NO_3 in serum of control rats, dehydroepiandrosterone (DHEA) (4 mg/21 days), ischemia/reperfusion (I/R), and DHEA (4 mg/21 days) plus I/R groups. Ischemia lasted 30 minutes, followed by 6 hours of reperfusion. Data are mean $\pm$ SD ($N = 8$ to 10). *$P < 0.05$ vs. control (C); †$P < 0.05$ vs. I/R.

linked secondary antibody and diaminobenzidine were used to detect specific binding. No positive staining was detected when tissue from the same animals was processed without primary antibody, indicating the absence of nonspecific binding under the conditions applied. The percentage of tubules with positive immunostaining out of 100 superficial cortex tubules was calculated.

Statistical analyses

All values in text and figures are expressed as means $\pm$ SD of determinations for all rats in the group ($N = 8$ to 10). For histologic scoring, results are expressed as means $\pm$ SEM. Data were analyzed using analysis of variance (ANOVA) followed by Bonferroni's post test and a P value below 0.05 was considered to be significant [36].

RESULTS

Oxidative stress parameters

Monolateral renal I/R (30 minutes/6 hours) determined a significant increase in pro-oxidant species in kidney tissue. Figure 1 shows high hydrogen peroxide levels in the cytosol obtained from kidney homogenate and high serum nitrite/nitrate levels in I/R rats compared with values for control and sham-operated animals. Sham-operated rats did not differ from control rats (data therefore omitted). The increased levels of both pro-oxidant species were reduced when DHEA was given for 21 days prior to I/R.

Cytosolic GSH peroxidase, catalase, and SOD activities were evaluated as indicators of antioxidant tissue defenses (Table 1). Renal I/R produced a significant reduction of all antioxidant enzyme activities analyzed. When DHEA was administered to rats before I/R, antioxidant activities were normalized.

Cytosolic renal TNF-α levels are in Figure 2. I/R rats showed an increase of cytosolic TNF-α levels versus DHEA plus I/R rats and also versus control rats. TNF-α

has been reported to modulate iNOS activity [13]. Figure 2 also reports iNOS levels determined by Western blot analysis. The iNOS level doubled in I/R rats versus control rats. In the DHEA plus I/R rats, iNOS was less expressed (about 70%) than in I/R rats without DHEA.

Moreover, renal I/R caused a significant increase in the cytosolic concentration of HNE, a toxic end product of lipid peroxidation, compared to controls (Fig. 3). The high HNE levels induced by I/R were reduced by DHEA pretreatment, but DHEA alone did not alter HNE levels versus control values. No difference was detected between control and sham-operated rats.

Renal functionality

Rats subjected to I/R showed a significant enhancement in serum creatinine levels compared to control and sham-operated rats. Renal dysfunction was also confirmed by the low Na/K-ATPase activity observed in the crude particulate fraction (Fig. 4) suggesting an impairment of ATP-dependent activities. Pretreatment with DHEA significantly reduced creatinine levels in the serum (Fig. 4) and restored ATPase activity to the control value (Fig. 4). Control and sham-operated rats did not differ for any of the parameters analyzed.

Morphologic and immunohistochemical studies

After 6 hours of reperfusion, the proximal convoluted tubules from the superficial and middle cortex showed different degrees of damage. The average score over 20 tubules was control and sham-operated rats = 0; DHEA = 0; I/R = 2.30 $\pm$ 0.40; and DHEA + I/R = 1.90 $\pm$ 0.10. DHEA pretreated rats showed a slight, statistically non-significant reduction of damage versus vehicle-treated animals (Fig. 5). Intense immunohistochemical staining for HNE-modified protein was observed in the cytosol of the damaged epithelial tubular cells after 30 minutes of ischemia and 6 hours of reperfusion (Fig. 6A). In contrast, HNE protein was slightly decreased when DHEA was administered before I/R (Fig. 6B). No HNE-modified proteins were detected in the sham-operated rats.

DISCUSSION

Renal ischemia followed by reperfusion determines oxidative stress, as shown by elevated levels of pro-oxidant reactive species and loss of antioxidant systems. In the kidney of rats subjected to I/R we found an increased level of H_2O_2 and reduced antioxidant enzyme activities, as well as GPX, catalase, and SOD. Moreover, we observed an increase in TNF-α and iNOS content, associated with an increased NO_2/NO_3 content. This study shows that DHEA administration completely prevents the changes in defense antioxidant mechanism and TNF-α and iNOS expression in kidneys of I/R rats, while having a significant but more limited effect on HNE and renal

Table 1. Glutathione peroxidase (GSH-PX) catalase and Cu/Zn superoxide-dismutase activities in kidney cytosol of control and dehydroepiandrosterone (DHEA)-treated rats subjected to ischemia/reperfusion (I/R) (30 minutes/6 hours)

	Control	DHEA	I/R	DHEA + I/R
GSH-PX *μmol NADPH/min/mg protein*	253.0 ± 38.2	272.0 ± 60.8	192.5 ± 7.8^a	226.3 ± 28.1^b
Catalase *nmol/mg protein*	3.32 ± 0.27	3.26 ± 0.15	2.53 ± 0.11^a	3.34 ± 1.67
Suporoxide dismutase *U/mg protein*	103.2 ± 2.4	116.2 ± 30.9	47.5 ± 8.9^a	102.8 ± 8.0^b

Data are mean $\pm$ SD ($N = 8$ to 10).
[a]$P < 0.05$ vs control; [b]$P < 0.05$ vs. I/R.
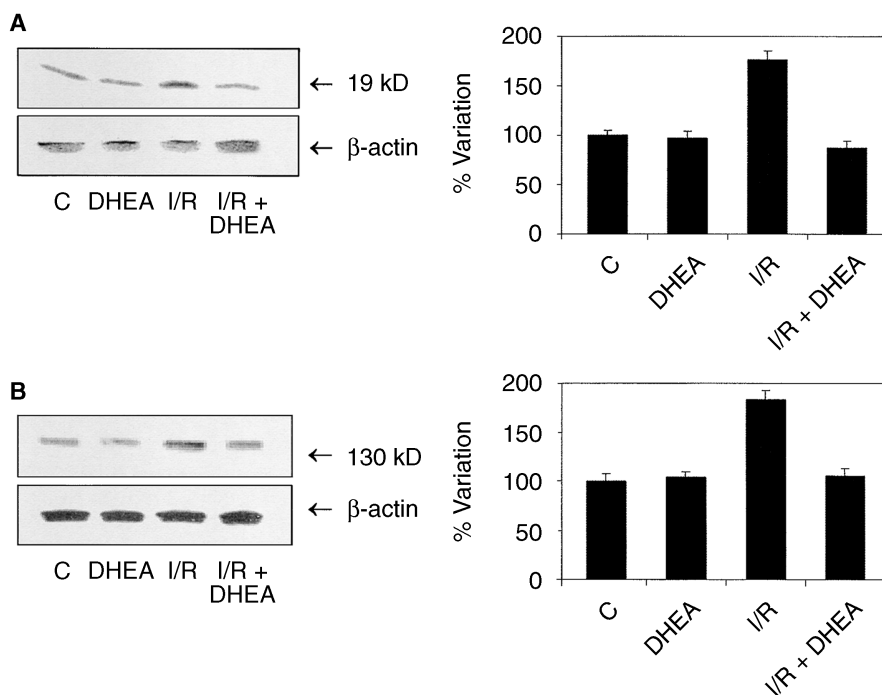
Fig. 2. Western blot of representative experiment showing tumor necrosis factor-α (TNF-α) (*A*) and inducible nitric oxide synthase (iNOS) (*B*) contents in the cytosol of control rats, dehydroepiandrosterone (DHEA) (4 mg/21 days), ischemia/reperfusion (I/R), and DHEA (4 mg/21 days) plus I/R groups. Ischemia lasted 30 minutes, followed by 6 hours of reperfusion. The histogram shows the relative densities of bands compared with β-actin. Data are mean $\pm$ SD of 8 to 10 rats per group.

Na/K-ATPase activity. This different effectiveness on the various parameters might be due to the specific DHEA dose used. In view of the chronic clinical use of DHEA, we treated rats with the lowest DHEA dose that exerts a significant antioxidant effect [7, 23], considering that higher doses possess a pro-oxidant effect (possibly via peroxisomal proliferation) [37, 38].

Oxidative stress increases TNF-α production [11], which, activating nuclear factor-κB (NF-κB), in turn induces TNF-α expression. DHEA can affect TNF-α production at both levels, since it reduces reactive species and modulates NF-κB activation [7, 23]. TNF-α, a pro-inflammatory cytokine, dramatically induces iNOS mRNA in epithelial tubular cells, and this is associated with increased nitric oxide production. Nitric oxide plays an ambiguous role in I/R tissue. It has been implicated in the regulation of renal vascular tone and tubular sodium transport, protecting the ischemic tissue during reperfusion [39, 40]. On the contrary, nitric oxide has also been found to be directly toxic in isolated rat proximal tubule segments exposed to hypoxia and reoxygenation [41]

and high levels of nitric oxide have also been observed in several renal dysfunction models [42]. The deleterious effect of increased nitric oxide in reperfused kidney might be greatly enhanced by a simultaneous increase in superoxide radicals. In the presence of superoxide, nitric oxide interacts with superoxide to form peroxynitrate radicals, an important agent that can cause oxidative and DNA damage [43].

DHEA treatment acts on both nitric oxide production and on the oxidative balance, thus reducing the formation of peroxynitrate radicals. The ability of DHEA to reduce the pro-oxidant species and to restore antioxidant levels has already been reported [21–23]; DHEA treatment in streptozotocin-treated rats reduces thiobarbituric acid reaction substance and fluorescent chromolipid and restores glutathione levels in the kidney as well as the enzymatic activity of catalase, SOD, and glutathione peroxidase (GSH-PX) [44]. Moreover, when DHEA is given to diabetic rats subjected to cerebral I/R, it exerts a beneficial effect against oxidative stress and ameliorates several markers of neuronal damage induced by free radi-
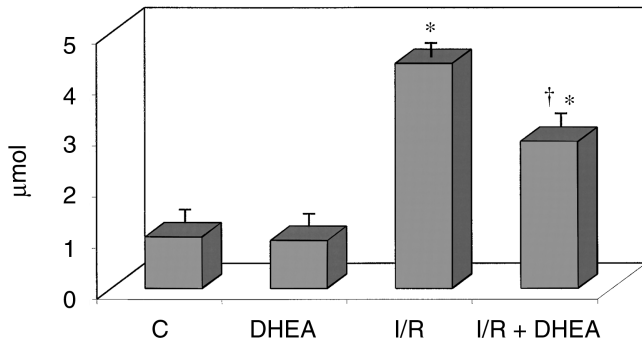
Fig. 3. Concentration of hydroxynonenal (HNE) in kidney cytosolic fraction of control rats, dehydroepiandrosterone (DHEA) (4 mg/21 days), ischemia/reperfusion (I/R), and DHEA (4 mg/21 days) plus I/R groups. Ischemia lasted 30 minutes, followed by 6 hours of reperfusion. Data are means ± SD ($N = 8$ to 10). *$P < 0.05$ vs. control (C); †$P < 0.05$ vs. I/R.
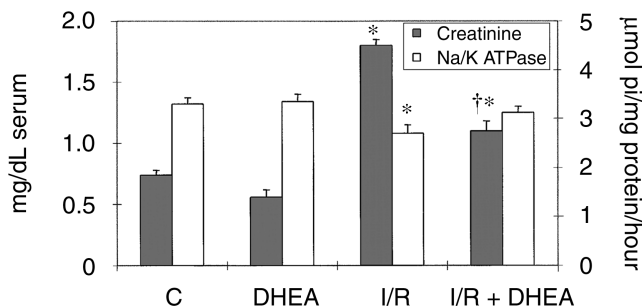

Fig. 4. Levels of creatinine in serum and Na/K ATPase activity in the crude particulate fraction of control rats, dehydroepiandrosterone (DHEA) (4 mg/21 days), ischemia/reperfusion (I/R), and DHEA (4 mg/21 days) plus I/R groups. Ischemia lasted 30 minutes, followed by 6 hours of reperfusion. Data are means ± SD ($N = 8$ to 10). *$P < 0.05$ vs. control (C); †$P < 0.05$ vs. I/R.
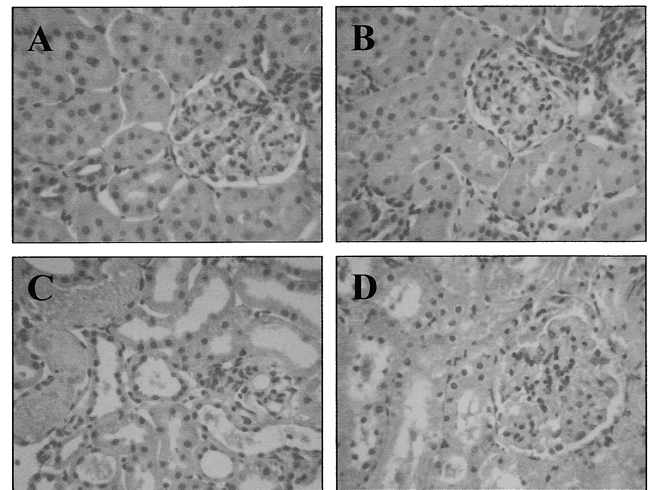

Fig. 5. Morphologic studies of kidney tissue of control rats, dehydroepiandrosterone (DHEA) (4 mg/21 days), ischemia/reperfusion (I/R), and DHEA (4 mg/21 days) plus I/R groups. Ischemia lasted 30 minutes, followed by 6 hours reperfusion. (*A*) Control. (*B*) DHEA. (*C*) I/R. (*D*) I/R + DHEA (hematoxylin-eosin stain, ×250). (A and B) Compatible with normal histology. (C and D) In contrast with DHEA-treated rats, the proximal tubules appear more dilatated and cell debris and cast formation are present in their lumen.
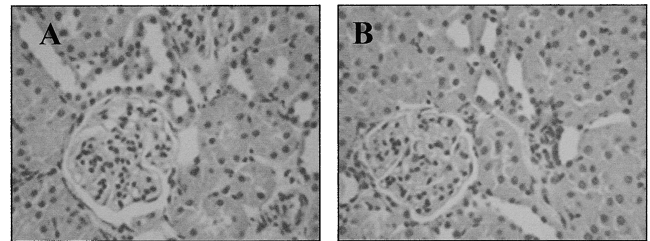

Fig. 6. Immunhistochemistry for hydroxynonenal (HNE) protein adducts on kidneys submitted to 30 minutes of ischemia followed by 6 hours of reperfusion. (*A*) Ischemia/reperfusion (I/R). (*B*) I/R plus dehydroepiandrosterone (DHEA)-treated (hematoxylin counterstained, ×400).

cals [7]. Here we show that besides improving the oxidative balance, DHEA protects lipid membranes against degradation, as is shown by the reduction in HNE concentration as well as by the restoration of Na/K-ATPase activity. Decreased renal Na/K-ATPase activity has been found in diabetic rats; I/R likewise reduced Na/K-ATPase activity, causing a loss membrane exchange functions. Here we show that DHEA significantly prevents loss of ATPase activity in I/R rats; we previously observed that DHEA counteracts the increase of hydroperoxyeicosatetranoic acids (HPETEs) in the kidney of diabetic rats [22]. HPETEs are potent inhibitors of Na/K-ATPase activity [45] and contribute to radical mediated kidney damage. We suggest that, also in I/R, HPETE reduction induced by DHEA might be one of the mechanisms involved in protecting Na/K-ATPase activity. Yorek et al [21] have suggested that DHEA might protect Na/K-ATPase activity through activation of mitogen-activated protein kinase and tyrosinase activity [46]. Although this possibility cannot be ruled out, a direct effect of DHEA

on Na/K-ATPase protein expression, at the doses used here, is unlikely since we did not observe any increase of Na/K-ATPase activity in the nonischemic kidney of rats treated with DHEA. Serum creatinine is widely used as a parameter to evaluate both glomerular filtration and tubular reabsorption. Here the protective effect of DHEA against I/R injury is confirmed by the improvement of creatinine levels. Furthermore, the DHEA group showed less evident morphologic damage. Hydropic degeneration of proximal convolute tubules appears to be reduced in DHEA pretreated rats. Recent studies indicate that, besides endothelial cells, the tubular epithelial cells are able to produce large amounts of nitric oxide [47]. The observed reduction of iNOS expression might suggest that, also in tubular cells, the decrease of both reactive nitrogen species and ROS contributes to the

protective effect of DHEA, reducing the production of the highly toxic peroxynitrate.

The mechanisms by which DHEA pretreatment protects the kidney against I/R-induced damage are not yet fully understood. DHEA becomes effective against oxidative injury of the brain induced by I/R only after more than 2 weeks of treatment [23]. One possibility is that inserting DHEA into the lipid membrane makes it more resistant to oxidative stress [48]. 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. DHEA has also been reported to change the fatty acid composition of mytochondrial membrane phospholipids in rats [49]. However, it is also possible to speculate on many other mechanisms, such as changes in the level of other substances that attenuate oxidative stress but are yet to be detected in this role. Modulation of NF-κB activation might play a pivotal role in the protective effect of DHEA [23]. TNF-α/NF-κB activation interplay occurs in oxidative stress [50]. Here we show an important reduction of the TNF-α level in DHEA-treated rats, thus TNF-α–dependent genes are not activated and tissue damage is reduced. Moreover, we suggest that another mechanism known to contribute to oxidative damage (i.e. peroxynitrite production) may be inhibited by DHEA [51]. DHEA treatment reduces both radical reactive species and nitric oxide production in I/R and thus further counteracts oxidative damage. We cannot rule out that some of the effects of DHEA on kidney injury and function might be due to its metabolites. Hayashi et al [52] showed that about 50% of the total antiatherosclerosis effect of DHEA (pharmacologic doses that can give rise to biologically active amounts of estrogens) depends on estrogens. However, we have observed negligible variations of both 17-β-estradiol and testosterone concentrations in rats treated with 4 mg of DHEA [53]. We suggest that estrogen receptors might be directly activated by DHEA and adiol, as we found in rats bearing 7.12-dimethylbenz(a)anthroacene (DMBA)-induced mammary tumor [53].

However, the question of whether the effect of DHEA is due to DHEA itself, to its metabolites, or to a combination of both is still open, since the relevant molecule(s) and mechanism(s) are largely unknown. Whatever the active molecules and mechanisms involved, the data clearly demonstrate the ability of DHEA pretreatment in a rat model to reduce renal injury associated to I/R. These results are of some importance since currently there is no clinically effective therapy that prevents renal ischemic injury.

ACKNOWLEDGMENTS

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