0013-7227/06/$15.00/0
Printed in U.S.A.

Endocrinology 147(12):5967–5974
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doi: 10.1210/en.2006-0728

Oxidative Stress-Dependent Impairment of Cardiac-Specific Transcription Factors in Experimental Diabetes

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Oxidative stress plays a key role in the pathogenesis of diabetic cardiomyopathy, which is characterized by myocyte loss and fibrosis, finally resulting in heart failure. The study looked at the downstream signaling whereby oxidative stress leads to reduced myocardial contractility in the left ventricle of diabetic rats and the effects of dehydroepiandrosterone (DHEA), which production is suppressed in the failing heart and prevents the oxidative damage induced by hyperglycemia in several experimental models. DHEA was given orally at a dose of 4 mg/rat per day for 21 d to rats with streptozotocin (STZ)-induced diabetes and genetic diabetic-fatty (ZDF) rats. Oxidative balance, advanced glycated end products (AGEs) and AGE receptors, cardiac myogenic factors, and myosin heavy-chain gene expression were determined in the left ventricle of treated and untreated STZ-diabetic rats and ZDF rats. Oxidative stress induced by chronic hyperglycemia increased AGE and AGE receptors and led to activation of the pleoitropic transcription factor nuclear factor-κB. Nuclear factor-κB activation triggered a cascade of signaling, which finally led to the switch in the cardiac myosin heavy-chain (MHC) gene expression from the α-MHC isoform to the β-MHC isoform. DHEA treatment, by preventing the activation of the oxidative pathways induced by hyperglycemia, counteracted the enhanced AGE receptor activation in the heart of STZ-diabetic rats and ZDF rats and normalized downstream signaling, thus avoiding impairment of the cardiac myogenic factors, heart autonomic nervous system and neural crest derivatives (HAND) and myogenic enhancer factor-2, and the switch in MHC gene expression, which are the early events in diabetic cardiomyopathy. (*Endocrinology* 147: 5967–5974, 2006)

DIABETIC CARDIOMYOPATHY, THE leading cause of death in diabetic patients, is characterized by both systolic and diastolic dysfunction, due to reduced contractility, prolonged relaxation, and decreased compliance (1, 2). The development of diabetic cardiomyopathy is multifactorial. Putative mechanisms include metabolic disturbances, small vessel disease, autonomic dysfunction, insulin resistance, and myocardial fibrosis (3, 4). Recently interstitial fibrosis has been regarded as an important pathogenetic factor of the heart's impaired functional integrity (5); however, altered substrate supply and use by cardiac myocytes could be the primary injury in the pathogenesis of this specific heart muscle disease (6). Structural and functional impairment of myocytes, which occurs already in the first week of diabetes, precedes cardiac fibrosis and thus remains the key step in impairing contractile performance, finally inducing heart failure.

Evidence suggests that overproduction of superoxide by the respiratory chain and the consequent oxidative stress play a role in the pathogenesis of diabetic complications (7). The increase in advanced glycated end-product (AGE) formation is among the main mechanisms recruited by oxidative stress and involved in diabetic damage (8). Besides the direct toxic effects, AGEs work by interacting with their receptors, a heterogeneous class of molecules (9). AGE/AGE receptor ligands mediate the long-term effects on key cellular targets, playing a pivotal role in modulating tissue injury in diabetes (10). Interruption of free radical overproduction by antioxidants counteracts AGE formation (11). However, the conventional antioxidants used to prevent oxidative damage in diabetes have failed to achieve substantial results (12) because these scavenger species react in a stoichiometric manner. A compound of physiological origin that possesses multitargeted antioxidant properties is dehydroepiandrosterone (DHEA) (13, 14), a multifunctional steroid that prevents the tissue damage induced by hyperglycemia in several *in vivo* and *in vitro* models (15–17). Moreover, it has recently been reported that the human heart synthesizes DHEA, that DHEA production is suppressed in the failing heart, and that plasma levels of the sulfate conjugate of DHEA decrease in patients with chronic heart failure in proportion to the severity of the condition (18).

This study looked at the downstream signaling activated by the oxidative stress and possibly mediating tissue damage in the left ventricle of diabetic rats and the effects of DHEA treatment on AGE receptor activation, specific myogenic factor level, and the expression of myosin heavy-chain (MHC) genes.

First Published Online August 24, 2006

Abbreviations: AGE, Advanced glycated end products; DCFH, 2′,7′-dichlorofluorescin; dHAND, decidiuum HAND; DHEA, dehydroepiandrosterone; eHAND, extra embryonic tissues, HAND; GSH, glutathione; GSSG, difference between total GSH and GSH content; HAND, heart autonomic nervous system and neural crest derivatives; HNE, hydroxynonenal; MEF, myogenic enhancer factor; NFkB, nuclear factor-κB; MHC, myosin heavy-chain; MS, mass spectrometry; RAGE, AGE receptor; ROS, reactive oxygen species; STZ, streptozotocin; TNFα-R1, TNF-α receptor-1; ZDF, Zucker Diabetic Fatty.

***Endocrinology* is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.**

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Materials and Methods

Animal treatment

Male Wistar rats (Harlan-Italy, Udine, Italy) weighing 200–220 g and 7-wk-old Zucker Diabetic Fatty (ZDF) (GMU *fa/fa*; Charles River Laboratories, Calco-Lecco, Italy) rats were cared for in compliance with the Italian Ministry of Health Guidelines (no. 86/609/EEC) and the *Principles of Laboratory Animal Care* (National Institutes of Health no. 85-23, revised 1985). They were provided with Piccioni pellet diet (no. 48; Gessate Milanese, Italy) and water *ad libitum*. Hyperglycemia was induced in Wistar rats through a single injection of streptozotocin (STZ) (50 mg/kg body weight) in the tail vein. Three days later glycemia was measured with the Accu-Check Compact kit (Roche Diagnostics Gmbh, Mannheim, Germany).

Only rats with blood glucose levels above 18 mmol/liter entered the experimental protocols; normoglycemic rats were used as controls. On the fourth day after injection, both hyperglycemic and control rats began DHEA treatment. DHEA was administered for 21 d at 4 mg/d per rat: crystalline DHEA was dissolved in 1 vol of 95% ethanol, mixed with 9 vol of mineral oil and given daily by gastric intubation. Controls received vehicle alone. ZDF rats were treated with DHEA or vehicle alone for 21 d at 4 mg/d per rat. After 21 d control and STZ-diabetic rats, with or without DHEA (n = 10 per group), and ZDF rats, with or without DHEA, were anesthetized with 20 mg/kg body weight of Zoletil 100 (Virbac, Carros, France) and killed by decapitation. Blood was collected and the plasma isolated. Glycemia was evaluated as described above. The heart was isolated and weighed, and the left ventricle was homogenized to obtain different extracts.

Tissue extracts

Cytosolic and nuclear extracts were prepared as described by Meldrum *et al.* (19). Briefly, tissues were homogenized at 10% (wt/vol) in a Potter Elvehjem homogenizer (Wheaton, Millville, NJ) using a homogenization buffer containing 20 mmol/liter HEPES (pH 7.9), 1 mmol/liter $MgCl_2$, 0.5 mmol/liter EDTA, 1% Nonidet P-40, 1 mmol/liter EGTA, 1 mmol/liter dithiothreitol, 0.5 mmol/liter phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin, and 2.5 μg/ml leupeptin. Homogenates were centrifuged at 1000 $\times$ *g* for 5 min at 4 C. Supernatants were removed and centrifuged at 105,000 $\times$ *g* at 4 C for 40 min to obtain the cytosolic fraction. The pelleted nuclei were resuspended in extraction buffer containing 20 mmol/liter HEPES (pH 7.9), 1.5 mmol/liter $MgCl_2$, 300 mmol/liter NaCl, 0.2 mmol/liter EDTA, 20% glycerol, 1 mmol/liter EGTA, 1 mmol/liter dithiothreitol, 0.5 mmol/liter phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin, and 2.5 μg/ml leupeptin and incubated on ice for 30 min for high-salt extraction, followed by centrifugation at 15,000 $\times$ *g* for 20 min at 4 C. The resulting supernatants containing nuclear proteins were carefully removed and samples were stored at −80 C until use. Protein content was determined using the Bradford assay (20).

Total RNA was extracted from heart tissue using TRIzol reagent (Invitrogen, Groningen, The Netherlands) based on the method developed by Chomczynski and Sacchi (21).

Oxidative biochemical parameters

Hydroxynonenal (HNE) concentration was also determined on fresh cytosol fractions by the method of Esterbauer *et al.* (22). After extraction, an aliquot of cytosol was injected into an HPLC (Waters Associated, Milford, MA) Symmetry C_{18} column (5 mm, 3.9 $\times$ 150 mm). The mobile phase was acetonitrile/bidistilled water (42%, vol/vol). The HNE concentration was calculated by comparison with a standard solution of HNE of known concentration.

Reactive oxygen species (ROS) were measured using probe 2′,7′-dichlorofluorescin (DCFH) diacetate. DCFH-DA is a stable, nonfluorescent molecule that readily crosses the cell membrane and is hydrolyzed by intracellular esterases to nonfluorescent (DCFH), which is rapidly oxidized, in the presence of peroxides, to highly fluorescent 2′,7′-dichlorofluorescein. The 2′,7′-dichlorofluorescein is measured fluorometrically (23).

The antioxidant level was evaluated in terms of reduced and oxidized glutathione content by the method of Owens and Belcher (24). A mixture was directly prepared in cuvette: 0.05 mol/liter Na-phosphate buffer (pH 7.0); 1 mmol/liter EDTA (pH 7.0); 10 mmol/liter 5,5′-dithiobis (2-nitro-benzoic) acid plus an aliquot of the sample; reduced glutathione (GSH) content was evaluated after 2 min at 412 nm and expressed as micrograms per milligram protein. Suitable volumes of diluted glutathione reductase and of β-nicotinamide adenine dinucleotide phosphate reduced tetrasodium salt were then added to evaluate the total GSH level. The difference between total GSH and GSH content represents the GSSG content (expressed as micrograms per milligram protein); the ratio between GSSG content and GSH is considered a good parameter of antioxidant status.

AGE detection (pentosidine) with HPLC-mass spectrometry (MS)

Plasma samples (200 μl) were treated with 6 M hydrochloric acid for 2 h at 40 C and then centrifuged (4000 rpm) (25); 20 μl of supernatant were injected.

A Thermo-Finnigan surveyor instrument (Thermo Electron, Rodano, Italy), equipped with autosampler and PDA-UV 6000 LP detector, was used. MS analyses were performed using an LCQ Deca XP (Thermo Electron) plus spectrometer, with electrospray interface and ion trap as mass analyzer.

Chromatographic separations were run on a Polaris C18-A column (150 $\times$ 2 mm, 3 μm particle size) (Varian, Leinì, Italy). Flow rate was 200 $\mu l/min^{-1}$. Gradient mobile phase composition was adopted: 95:5 to 0:100 (vol/vol) 15 mM heptafluorobutanoic acid in water/methanol in 13 min.

The LC column effluent was delivered to a UV detector (200–400 nm) and then to the ion source, using nitrogen as sheath and auxiliary gas (Claind nitrogen generator apparatus; Lenno, Italy). The source voltage was set at 4.5 kV in the positive mode. The heated capillary was maintained at 200 C. The acquisition method used was previously optimized in the tuning sections for pentosidine quasimolecular ion (capillary, magnetic lenses, and collimating octapole voltages) to maximize sensitivity. Collision energy was chosen to maintain about 10% of the precursor ion. The tuning parameters adopted for the electrospray ionization source were the following: source current 80.00 μA; capillary voltage 3.00 V; tube lens offset 15 V; for ion optics, multipole 1 offset −5.25 V, intermultipole lens voltage −16.00 V, multipole 2 offset −9.00 V. Mass spectra were collected in tandem MS mode: MS^2 of (+) 379 m/z with 33% collision energy in the range 100–400 m/z.

TNF-α

TNF-α was determined in cytosol using a specific enzyme immunoassay (rat TNF-α ELISA kit; Diaclone Research, Besancon, France), following the manufacturer's instructions.

Western blotting

dHAND, eHAND, receptor of AGE (RAGE), and galectin-3 were detected on cytosolic extracts. Myogenic enhancer factor (MEF)-2 and nuclear factor-κB (NFkB)-p65 were detected on nuclear extracts by Laemmli's method (26). Equal amounts of proteins (60 μg) were separated on 10% SDS-polyacrylamide gels and then blotted on nitrocellulose membranes (Amersham Biosciences, Braunschweig, Germany). The membranes were blocked with 5% (wt/vol) nonfat dry milk in 5 mM Tris-HCl (pH 7.4) containing 200 mM NaCl and 0.05% (vol/vol) Tween 20 for 1 h at 25 C; incubated overnight with rabbit polyclonal antibodies against dHAND, eHAND, RAGE, and MEF-2 and mouse monoclonal antibodies against galectin-3 and NFkB-p65 (Santa Cruz Biotechnology, Santa Cruz, CA); and reacted with peroxidase-labeled secondary antibodies (Santa Cruz Biotechnology) in Tris-buffered saline-Tween containing 2% (wt/vol) nonfat dry milk. Immunoreactive proteins were detected through the chemiluminescence assay (Amersham) and subsequent exposure to film for 2–10 min. Anti-α-actinin and antilamin B1 antibodies served as loading controls respectively for cytosolic and nuclear proteins. Specific bands were quantified by densitometry using an analytic software (Multi-Analyst; Bio-Rad, Munchen, Germany), and the net intensity of bands in each experiment was normalized for the intensity of the corresponding α-actinin or lamin B1 band before comparison between control and treated samples.

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RT-PCR

RNA was reverse transcribed at 42 C for 40 min, using avian myeloblastosis virus reverse transcriptase (Finnzymes, Espoo, Finland) in the presence of oligo-dT primer (Invitrogen). The PCR contained 5 μl of 10 × PCR buffer, 1 μl reverse transcription product, 0.2 mM dNTP (Finnzymes), 1.25U *Taq* DNA polymerase (Finnzymes), 50 pmol of sense and antisense primers. Primers included: α-MHC sense, 5′-GGA CCA CCC ATC CTC ACT TT; antisense, 5′-AGC CTC TCA TCT CGC ATC TC; β-MHC sense, 5′-ACC GCT GAG ACA GAG AAT GG; antisense, 5′-GGG TTG GCT TGG ATG ATT T; TNF-α receptor-1 (TNFα-R1)sense, 5′-CCT GAT TTC CAT CTA CCT CTG ACT; antisense 5′-GAA ATG CGT CTC ACT CAG GTA G; cyclophilin sense, 5′-ACG CCG CTG TCT CTT TTC; antisense, 5′-TGC CTT CTT TCA CCT TCC.

Amplification was carried out as follows: for α-MHC and β-MHC, 25× (95 C, 30 sec; 58 C, 30 sec; 72 C, 30 sec). For TNFα-R1: 30× (94 C, 30 sec; 58 C, 30 sec; 72 C, 30 sec). For cyclophilin: 25× (94 C, 1 min; 50 C, 1 min; 72 C, 1 min). PCR products were electrophoresed on 1.5% agarose gel in the presence of ethidium bromide. Gels were photographed and analyzed with 1D Image Analysis software (Kodak, Rochester, NY). The net intensity of bands in each experiment was normalized for the intensity of the corresponding cyclophilin band before comparison between control and treated samples.

Statistical analysis

All results are presented as means ± SD. Differences between means were analyzed for significance using one-way ANOVA with the Bonferroni posttest (27).

Results

At the end of 3 wk, the STZ-diabetic rats showed a body weight lower than controls; DHEA treatment reversed this reduction in the STZ-diabetic group (control group, 245.6 ± 9.8 g; STZ, 220.9 ± 9.9 g; STZ+DHEA, 241.8 ± 11.8 g; see Table 1 for statistical significance). The heart weight normalized for body weight was also significantly less in diabetic rats than controls, and DHEA treatment reversed this reduction (control group, 3.68 ± 0.21 mg/g; STZ, 2.84 ± 0.18 mg/g; STZ+DHEA, 3.25 ± 0.26 mg/g; see Table 1 for statistical significance). Body weight and heart weight of ZDF rats treated with DHEA did not differ from those of untreated ZDF rats (data not shown).

Glycemia evaluated 3 d after STZ injection was maintained high throughout the experimental protocol with or without DHEA treatment (control group, 6.9 ± 0.9 mmol/liter; STZ, 21.4 ± 2.3 mmol/liter; STZ+DHEA, 20.6 ± 1.5 mmol/liter; see Table 1 for statistical significance). Plasma levels of glucose in ZDF (24.41 ± 3.09 mmol/liter) and ZDF-plus-DHEA rats (25.50 ± 2.35 mmol/liter) were unmodified after 21 d of treatment. DHEA levels in the plasma after 21 d of treatment reached values similar to normal human values (28).

Oxidative balance

Both ROS and TNF-α levels (Table 1) in STZ-diabetic rats were significantly higher than in controls. DHEA treatment in STZ-treated rats ameliorated the oxidative imbalance, reducing the ROS and restoring the level of antioxidant. The ratio GSSG/GSH increased *vs.* the STZ-alone group.

The concentration of HNE, an end product derived from lipid peroxidation, also increased significantly in STZ-diabetic rats, and DHEA treatment restored this value to control levels (control group, 0.86 ± 0.06 μmol/liter; DHEA, 0.76 ± 0.24 μmol/liter; STZ, 1.81 ± 0.51 μmol/liter; STZ+DHEA, 1.22 ± 0.58 μmol/liter; see Table 1 for statistical significance). DHEA treatment in ZDF rats determined a good reduction of oxidative stress parameters, compared with untreated ZDF rats (data not shown).

AGE and RAGE

The concentration of pentosidine was measured in plasma from control, STZ-alone, and STZ-plus-DHEA rats and ZDF and ZDF-plus-DHEA rats. In STZ-diabetic rats, pentosidine levels were doubled from those of control rats; DHEA treatment of STZ rats prevented this increase. DHEA alone produced no change (Table 1). Also ZDF rats, with or without DHEA, showed a similar pattern with respect to STZ models (data not shown).

Figure 1A shows the RAGE content in the cytosol of the left ventricle of STZ-diabetic rats with and without DHEA (4 mg for 21 d). DHEA treatment in STZ rats restored the content of RAGE to a significant extent. The expression of RAGE, evaluated with PCR (Fig. 1C), was doubled in STZ rats; this increase was significantly reversed in STZ rats supplemented with DHEA. DHEA-treated ZDF rats (Fig. 1, B and panel D) showed a decrease of content and expression of RAGE, compared with untreated ZDF rats.

Galectin-3 content was also significantly increased in the STZ-diabetic heart (STZ-rats 3.5-fold *vs.* control rats) and was restored to control levels in DHEA-treated rats (STZ+DHEA halved *vs.* STZ) (Fig. 2).

Transcription factors

NFkB-p65 protein levels in nuclear extract from the left ventricle were determined by Western blotting (Fig. 3). STZ-diabetic rats showed a marked increase in NFkB-p65 protein content, explained by its translocation from the cytosol. DHEA treatment modulated NFkB translocation: NFkB-p65 protein level in the nucleus was significantly lower after DHEA treatment (Fig. 3A). Also in ZDF rats, the translocation of NFkB-p65 in the nucleus was lower after DHEA treatment (Fig. 3B).

Figure 4 reports the content of cardiac-specific transcription factors, MEF-2 in nuclear extracts (Fig. 4A),

TABLE 1. ROS, GSSH to GSH ratio, and TNF-α in cytosol of left ventricle and pentosidine in plasma of control, STZ-treated rats with or without DHEA (4 mg per 21 d)

	ROS (U.F./mg protein)	GSSG to GSH (ratio)	TNF-α (pg/mg protein)	Pentosidine (μM)
Control	122.99 ± 10.62	0.64 ± 0.05	50.23 ± 2.90	0.255 ± 0.114
Control+DHEA	114.15 ± 5.31	0.64 ± 0.07	53.80 ± 2.22	0.288 ± 0.097
STZ	307.62 ± 33.82[a]	1.23 ± 0.24[a]	72.90 ± 6.70[a]	0.528 ± 0.073[a]
STZ+DHEA	147.62 ± 11.48[b]	0.74 ± 0.20[b]	53.50 ± 7.40[b]	0.287 ± 0.081[b]

Data are means ± SD of nine to 10 rats per group. U.F., Arbitrary unit of fluorescence.
[a] Statistical significance: *vs.* control, $P < 0.05$.
[b] Statistical significance *vs.* STZ, $P < 0.05$.

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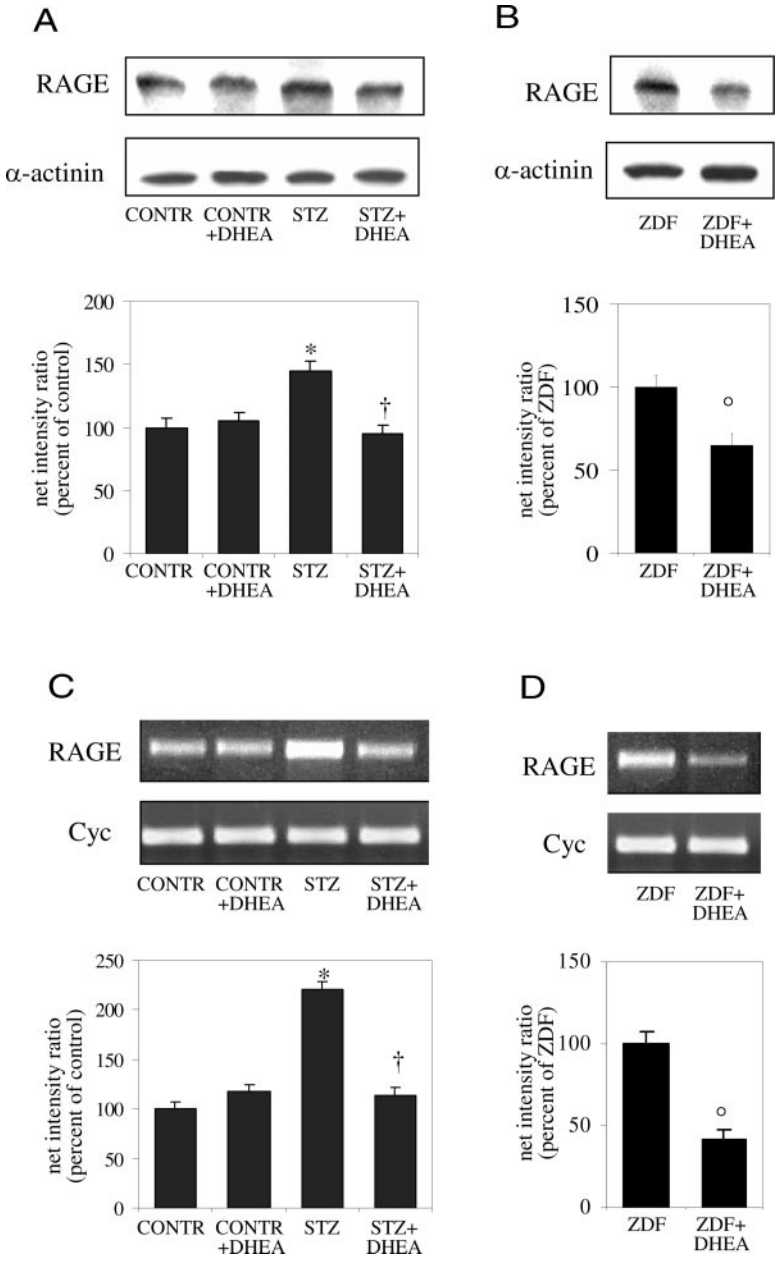

FIG. 1. RAGE content in heart cytosol from control (CONTR) and STZ-diabetic rats (A) and ZDF rats (B), treated or not with DHEA (4 mg per 21 d). The histograms represent the net intensity ratio with α-actinin and data are expressed as percentage variation *vs.* control value or ZDF value. RAGE expression in heart from control and STZ-diabetic rats (C) and ZDF rats (D), treated or not with DHEA (4 mg per 21 d). The histograms represent the net intensity ratio with cyclophilin (Cyc) and data are expressed as percentage variations *vs.* control value or ZDF value. Data are means ± SD of nine to 10 rats per group. *, Statistical significance *vs.* CONTR, $P < 0.05$; †, statistical significance *vs.* STZ, $P < 0.05$; °, Statistical significance *vs.* ZDF, $P < 0.05$.

dHAND and eHAND in the cytosol (Fig. 4B) of the left ventricle in control, DHEA-alone, STZ-alone, and STZ-plus-DHEA groups. The primary antibody against MEF-2 binds to both MEF-2A and MEF-2C isoforms, which were identified by their different molecular masses, respectively, 70 and 60 kDa. MEF-2 and HAND bands were significantly reduced in diabetic rats *vs.* controls. DHEA treatment of STZ rats significantly reversed the reduction of all transcription factors, as the histograms show. ZDF rats with or without DHEA showed a pattern similar to STZ models (data not shown).

TNF-α and TNFα-R1

TNFα-R1 expression in the left-ventricle from control rats and STZ-treated rats with and without DHEA is reported in Fig. 5. As for TNF-α protein level (Table 1), TNFα-R1 expression was markedly increased in STZ rats but was close to control values in DHEA-treated diabetic rats.

α-MHC and β-MHC

PCR analysis showed expression of two isoforms of the MHC protein, α-MHC and β-MHC, in the left ventricle of control, control plus DHEA, STZ-alone, and DHEA-plus-STZ rats. Figure 6 shows the decrease in α-MHC and the increase in β-MHC isoforms in STZ-rats; DHEA treatment of diabetic rats modulated this switch in STZ-rats (Fig. 6A) and in ZDF-rats (Fig. 6B).

Discussion

We here show that STZ-induced diabetes leads to a loss in cardiac mass (29, 30), which is prevented by DHEA treat-

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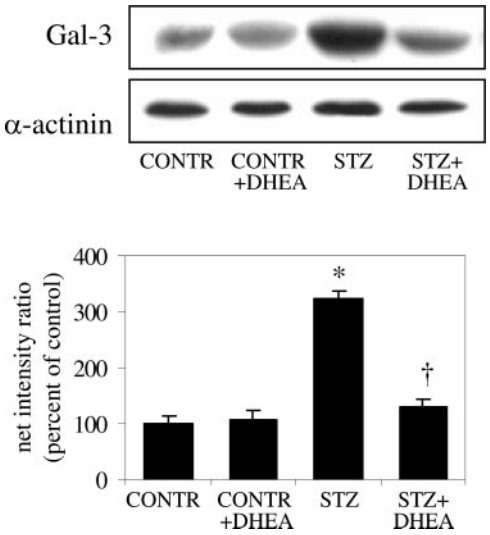

FIG. 2. Galectin-3 content in heart cytosol from control (CONTR) and STZ-diabetic rats, treated or not with DHEA (4 mg per 21 d). The histogram represents the net intensity ratio with α-actinin and data are expressed as percentage variations *vs.* the control value. Data are means ± SD of nine to 10 rats per group. *, Statistical significance *vs.* CONTR, $P < 0.05$; †, statistical significance *vs.* STZ, $P < 0.05$.

ment. We also show that diabetes, inducing oxidative imbalance, increases AGE levels, up-regulates both RAGE and galectin-3 levels, and increases TNF-α and TNFα-R1 levels, and we suggest that these oxidative-dependent events might impair the level of cardiac-specific transcription factors, thus also impairing the expression of the myosin heavy chain genes in the rat left ventricle. DHEA treatment, which prevents the thioredoxin imbalance, counteracts activation of downstream signaling, and protects the rat heart from myofibrillar remodeling, the early event of diabetic cardiomyopathy. This protection exerted by DHEA has been confirmed in ZDF rats.

MHCs are the heart's molecular motor, and its contractile properties depend to a great extent on the isoform composition of MHC proteins. A switch in MHC isoform composition has been reported to cause reduced contractile velocity and energy expenditure (31). In man, as well as in animals, loss of α-MHC content, which is expressed exclusively in the myocardium, has been reported to be responsible for the reduced myocardial contractility during heart failure (32) and in diabetes (33). We show here a net reduction in α-MHC expression and a marked increase in β-MHC expression in the left ventricle in diabetic rats. The mechanisms by which MHC synthesis is impaired can involve multiple transcription factors, including MEF-2 and HAND family: we demonstrate that eHAND, dHAND, and MEF-2 levels are reduced in the left ventricle of diabetic rats and that their reduction is prevented by DHEA treatment. MEF-2, which has been implicated in pathological remodeling of the adult human heart in response to stress signaling (34), cooperates with HAND to bind to the consensus E-box sequence and activate expression of α-MHC (35, 36). HAND isoforms are myogenic basic helix-loop helix proteins that recognize a DNA sequence E-box (CAnnTG) in cardiac target genes (35). It is noticeable that the down-regulation of eHAND is characteristic of human dilated or ischemic cardiomyopathy, whereas it is never observed in hypertrophic cardiomyopathy (37). In our model of STZ-diabetic cardiomyopathy, in which the heart weight is reduced, we observed a down-regulation of eHAND transcription factor, which is prevented by DHEA treatment. dHAND is also down-regulated in STZ-rats, suggesting that multiple transcription factors

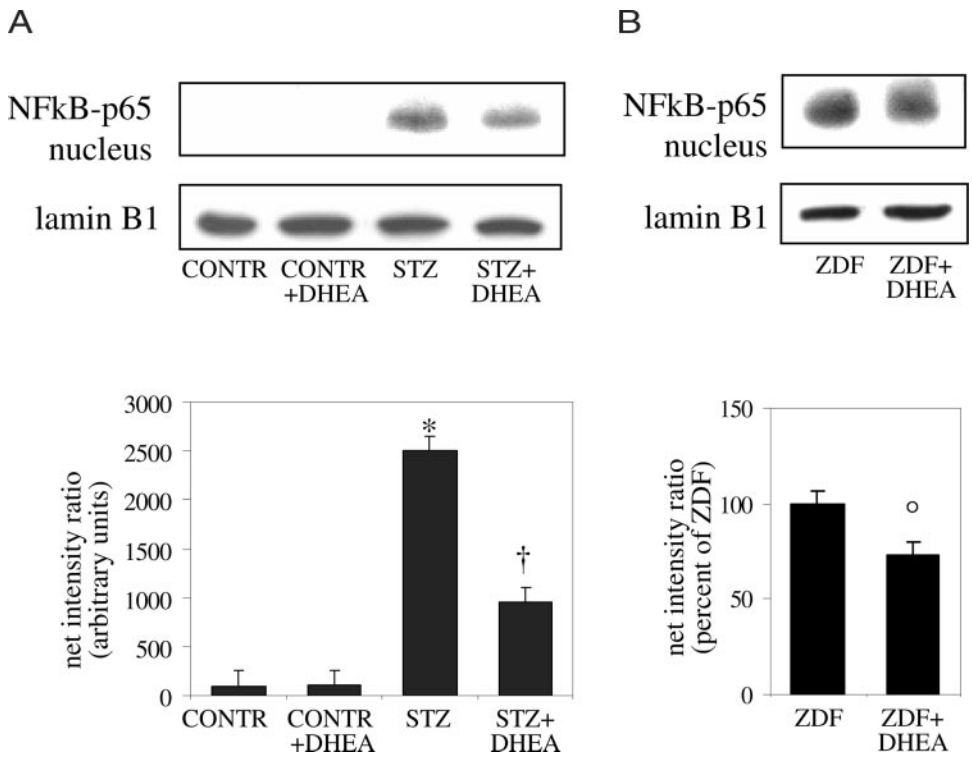

FIG. 3. NFkB-p65 content in heart nucleus from control (CONTR) and STZ-diabetic rats (A) and ZDF rats (B), treated or not with DHEA (4 mg per 21 d). The histograms represent the net intensity ratio with nuclear lamin B1; data are expressed as percentage variations *vs.* control value or ZDF value. Data are means ± SD of nine to 10 rats per group. *, Statistical significance *vs.* CONTR, $P < 0.05$; †, statistical significance *vs.* STZ, $P < 0.05$; °, statistical significance *vs.* ZDF, $P < 0.05$.

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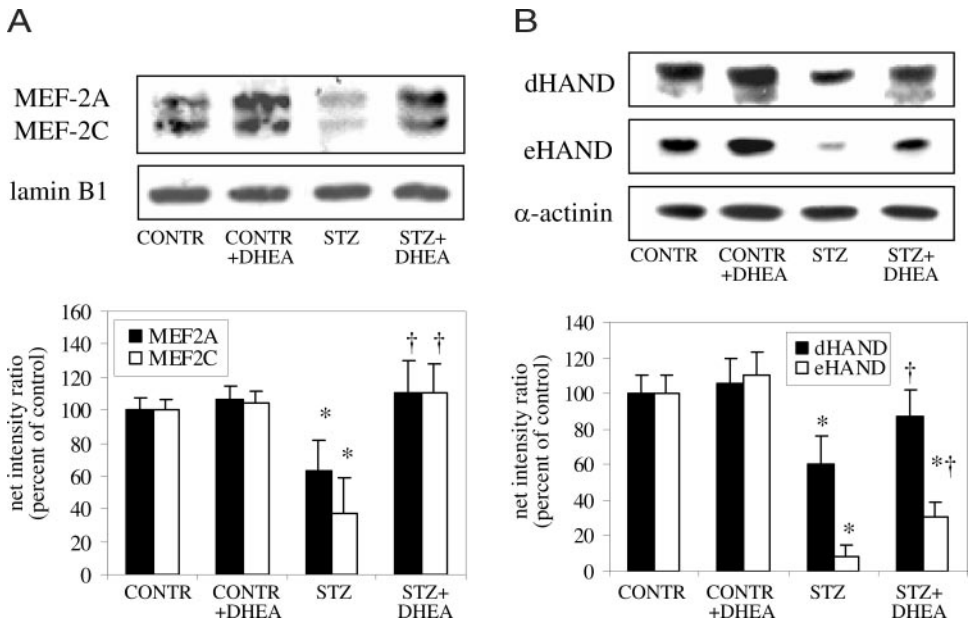

FIG. 4. MEF-2A and MEF−2C isoform content in nuclear extracts (A) and dHAND and eHAND content in cytosol fractions of heart (B) from control (CONTR) and STZ-diabetic rats, treated or not with DHEA (4 mg per 21 d). The histogram represents the net intensity ratio with lamin-B1 or α-actinin; data are expressed as percentage variations *vs.* control value. Data are means ± SD of nine to 10 rats per group. *, Statistical significance *vs.* CONTR, $P < 0.05$; †, statistical significance *vs.* STZ, $P < 0.05$.

belonging to the HAND family, in cooperation with MEF-2, guide the myosin heavy chain synthesis in diabetic cardiomyopathy.

We recently demonstrated that oxidative stress directly impairs myosin chain gene expression in the skeletal muscle of diabetic rats (16). The results reported here on oxidative balance and downstream signaling suggest that also in the left ventricle in diabetic rats oxidative stress contributes to the changes in myosin chain gene expression, leading to myofibrillar remodeling. In diabetes, AGEs, whose formation is closely correlated to oxidative stress (38) and which accumulate in the tissues of diabetic patients (39), participate in diabetes-induced myocardial structural changes (40). Besides their well-known direct toxicity, AGEs exert their detrimental effect by interacting and up-regulating their receptors, including RAGE and galectin-3 (41). This interaction involves the activation of transcription factors, such as NFkB, a major target of ROS. Activation of NFkB-dependent genes triggers several pathways, *i.e.* the production of proinflammatory cytokines, such as TNF-α, which is mainly involved in heart damage (39). TNF-α, in turn, activates NFkB and induces the RAGE gene, thus amplifying its detrimental effects on the diabetic heart (42–44). We show here that TNF-α is clearly augmented in the diabetic heart along with the redox imbalance, up-regulation of the RAGEs, and increased NFkB levels. We also show that TNFα-R1, which is mainly involved in the proinflammatory effects of TNF, is up-regulated, as we previously observed in hepatoma-bearing rats (45). In transgenic mice with cardiac-specific TNF overexpression (43), α-MHC is down-regulated and β-MHC is up-regulated, confirming the impact of TNF-α on the transcriptional regulation of MHC.

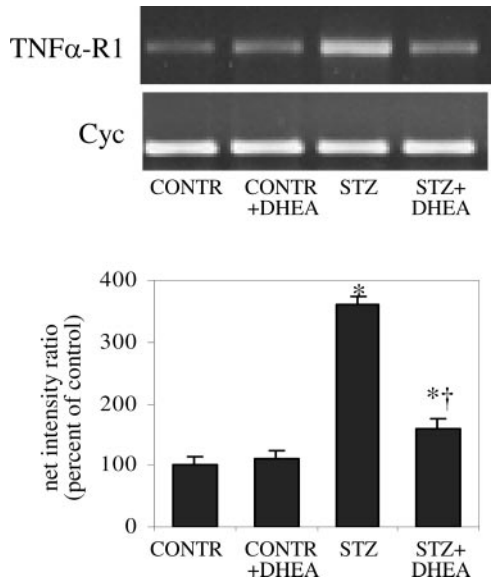

FIG. 5. TNFα-R1 expression in heart cytosol from control (CONTR) and STZ-diabetic rats, treated or not with DHEA (4 mg per 21 d). The histogram represents the net intensity ratio with cyclophilin (Cyc); data are expressed as percentage variations *vs.* control value. Data are means ± SD of nine to 10 rats per group. *, Statistical significance *vs.* CONTR, $P < 0.05$; †, statistical significance *vs.* STZ, $P < 0.05$.

The modulatory effect of DHEA on NFkB activation and the consequent TNF-α reduction might intervene in restoring levels of specific transcription factors to those observed in control animals and preventing the switch of the cardiac MHC gene expression, from α-MHC to a predominant β-MHC. We are aware that the findings presented cannot definitively prove the mechanistic relationship between oxidative stress and myocardial impairment. However, a correlation between systolic and diastolic myocardial dysfunction and oxidative stress has recently been reported in a highly selected group of uncomplicated type 2 diabetic patients (5), and the greater propensity for oxidative stress after myocardial infarction is associated with the development of heart failure (46).

We suggest that the improved redox balance is the chief mechanism underlying DHEA's beneficial effects on the heart (47). In previous studies we have shown that DHEA

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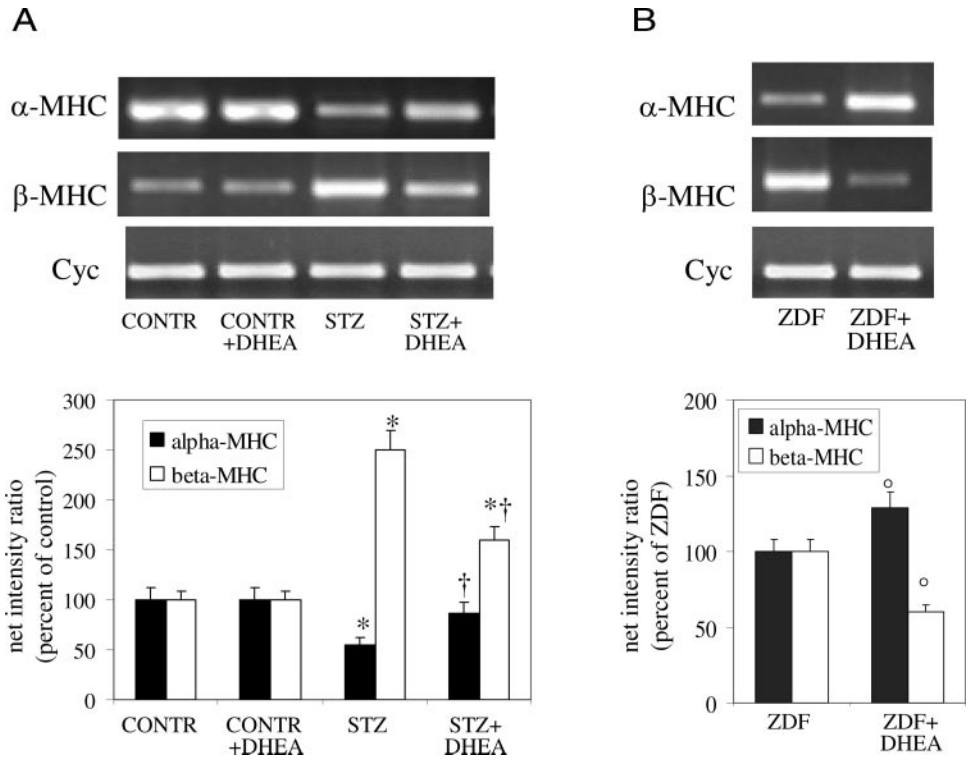

FIG. 6. α-MHC and β-MHC expression in heart cytosol from control (CONTR) and STZ-diabetic (A) and ZDF rat (B), treated or not with DHEA (4 mg per 21 d). The histograms represent the net intensity ratio with cyclophilin (Cyc); data are expressed as percentage variations *vs.* control value or ZDF value. Data are means ± SD of nine to 10 rats per group.

markedly reduces both ROS and TNF production induced in the kidney by ischemia/reperfusion (48) and that it modulates NFkB activation and counteracts RAGE up-regulation (49) in the hippocampus of diabetic rats. Moreover, the role of DHEA in the cardiovascular system (50) has been highlighted by the recent observation of DHEA production and CYP-17 gene expression, a key enzyme of DHEA synthesis, in human heart (51). More interestingly, it has been reported that, in man, DHEA production is suppressed in the failing heart and that, in rat cardiac fibroblasts, DHEA markedly attenuates production of collagen type I, counteracting cardiac fibrosis (52).

Several explanations have been proposed for the multitargeted antioxidant effects of DHEA, including its effect on catalase expression (53), up-regulation of the redox system (47), fatty-acid composition of cellular membranes, and cytokine production. However, the precise mechanisms remain to be clarified and additional nonantioxidant effects cannot be excluded. Whether the effect of DHEA is due to DHEA itself, its metabolites, or a combination of both remains unclear. However, we found negligible variations of either 17β-estradiol or testosterone concentration in rats treated with 4 mg DHEA (17). Nevertheless, we reported elsewhere that DHEA, but not a variety of other steroids including β-estradiol, 5-en-androsten-3-β,17β-diol, and dihydrotestosterone, protects bovine retinal capillary pericytes against glucose-induced lipid peroxidation (17).

In conclusion, the results of this study show that the endogenous steroid DHEA, by preventing activation of the oxidative pathways induced by hyperglycemia, counteracts the enhanced RAGE activation in the heart of STZ-diabetic rats and ZDF rats and normalizes downstream signaling, thus avoiding impairment of the cardiac myogenic factors HAND and MEF-2 and the switch in MHC gene expression, which are the early events in diabetic cardiomyopathy. These observations, together with the reported effect of DHEA on cardiac fibroblasts (52), point the way toward an additional therapeutic approach to diabetic cardiomyopathy.

Acknowledgments

Received June 1, 2006. Accepted August 16, 2006.

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This work was supported by Fondo per gli Investimenti della Ricerca di Base, Regione Piemonte, and Ministero Italiano Università e Ricerca.

Disclosure statement: The authors have nothing to disclose.

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