Dehydroepiandrosterone Administration Counteracts Oxidative Imbalance and Advanced Glycation End Product Formation in Type 2 Diabetic Patients

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OBJECTIVE — Dehydroepiandrosterone (DHEA) has been shown to prevent oxidative stress in several in vivo and in vitro models. This study aimed to evaluate the effects of DHEA administration on oxidative stress, pentosidine concentration, and tumor necrosis factor (TNF)-α/TNF-α receptor system activity in patients with type 2 diabetes.

RESEARCH DESIGN AND METHODS — Twenty patients were enrolled in the study and randomly assigned to the DHEA ($n = 10$) or placebo ($n = 10$) group. Twenty healthy sex- and age-matched subjects with normal glucose levels served as control subjects. DHEA was given as a single daily dose of 50 mg for 12 weeks.

RESULTS — Oxidative stress parameters were significantly higher in diabetic patients versus control subjects. Pentosidine levels, as well as soluble TNF receptor (sTNF-R)I and sTNF-RII, were also higher in diabetic patients. After DHEA, plasma levels of reactive oxygen species and hydroxynonenal dropped by 53 and 47%, respectively, whereas the nonenzymatic antioxidants glutathione and vitamin E increased (+38 and +76%, respectively). The same changes in oxidative parameters were detected in peripheral blood mononuclear cells (PBMCs). DHEA treatment also induced a marked decrease of pentosidine plasma concentration in diabetic patients (−50%). Moreover, the TNF-α/TNF-α receptor system was shown to be less activated after DHEA treatment, in both plasma and PBMCs.

CONCLUSIONS — Data indicate that DHEA treatment ameliorates the oxidative imbalance induced by hyperglycemia, downregulates the TNF-α/TNF-α receptor system, and prevents advanced glycation end product formation, suggesting a beneficial effect on the onset and/or progression of chronic complications in type 2 diabetic patients.

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The onset and progression of diabetes complications involves a complex interplay between ranges of pathogenic mechanisms. However, emerging evidence suggests that a single early phenomenon, i.e., the overproduction of superoxide by the respiratory chain, plays a key role in the pathogenesis of both microvascular and macrovascular chronic complications (1–3).

The production of advanced glycation end products (AGEs) is among the main mechanisms recruited by oxidative stress and is involved in the pathogenesis of tissue injury (3,4). AGEs progressively accumulate with time at the sites of diabetic microvascular disease and mediate tissue damage by activation of specific receptors at distant sites, via circulation (3,5). Moreover, the AGE/AGE receptor interaction, along with hyperglycemia-induced oxidative stress, possibly serves as a key activator of upstream kinases, leading to increased production of inflammatory cytokines thought to be involved in the progression of chronic diabetes complications (6).

Interruption of free radical overproduction by antioxidants counteracts AGE formation (2). Nevertheless, despite convincing experimental results, clinical trials with traditional antioxidants have been disappointing (7)—the activity of the antioxidants used in those trials is limited to scavenging already-formed oxidants and is stoichiometric. A compound of physiological origin that possesses multi-targeted antioxidant properties is dehydroepiandrosterone (DHEA), a multifunctional steroid that has been shown to prevent tissue damage induced by hyperglycemia in several in vivo and in vitro models (8,9). It also prevents the upregulation of AGE receptors observed in the hippocampus of streptozotocin-induced diabetic rats (10).

This study aimed to examine the effects of DHEA administration on oxidative stress, pentosidine (a marker of AGE-biogenesis) concentration, and tumor necrosis factor (TNF)-α/TNF-α receptors in patients with recently diagnosed type 2 diabetes, controlled with diet alone, and without any evidence of chronic complications, i.e., with the disease at a very early stage.

RESEARCH DESIGN AND METHODS — The study was approved by the ethics committee at our institution, and written informed consent was obtained from all recruited subjects.

It was a randomized, double-blind, placebo-controlled, small-scale study of 12-week duration.

The study group comprised 20 patients with recently diagnosed type 2 diabetes treated with diet alone, having good glycemic control, and taking no drugs potentially interfering with redox status. All patients were nonsmokers and showed no evidence of chronic diabetes complications. The control group comprised 20 healthy subjects, matched by sex and age, with normal glucose levels.

After consent, patients were randomly assigned to the DHEA ($n = 10$) or the placebo ($n = 10$) group. DHEA was purchased from DHEAPharma (Miami, FL) and given as a single daily oral dose of 50 mg at 0800 h for 12 weeks. The 10 patients enrolled in the placebo group received pills that were identical in appearance to the DHEA formulation. Compliance was checked by pill counts. At baseline and at the end of the treatment, all patients were subjected to complete physical examination and fasting blood samples collected to evaluate oxidative stress parameters, pentosidine levels, and TNF-α/TNF receptors.

Serum DHEA, DHEA sulfate, glucose, serum insulin, homeostasis model assessment, and A1C

Serum DHEA and DHEA sulfate (DHEAS) were determined by specific radioimmunoassay (Diagnostic System Laboratories, Oxford, U.K.). Fasting serum glucose was measured by the glucose oxidase method (HITACHI 911 Analyzer; Sentinel Ch., Milan, Italy), and fasting serum insulin by immunoradiometric assay (Radim S.p.A., Pomezia, Italy). A1C was determined by standardized affinity high-performance liquid chromatography (BioRad). The homeostasis model assessment (HOMA) of insulin resistance index was calculated as the product of basal glucose and insulin levels divided by 22.5 (11).

Cytosol extracts from PBMCs

To isolate peripheral blood mononuclear cells (PBMCs), heparin-anticoagulated blood was subjected to density-gradient centrifugation on Lymphoprep (Fresenius Kabi Norge, Oslo, Norway). The cytosolic extracts were prepared as reported elsewhere (12). PBMCs were resuspended in buffer containing 20 mmol/l HEPES, pH 7.9, 1 mmol/l MgCl$_2$, 0.5 mmol/l EDTA, 1% NP-40, 1 mmol/l EGTA, 1 mmol/l DTT, 0.5 mmol/l PMSF, 5 μg/ml aprotinin, and 2.5 μg/ml leupeptin, then

centrifuged at 1,000g for 5 min at 4°C. Supernatants were centrifuged at 105,000g at 4°C for 40 min to obtain cytosolic fraction, and protein content was determined.

Pentosidine

Plasma samples (200 μl) were treated with 6 mol/l hydrochloric acid for 2 h at 40°C and centrifuged (4,000 rpm), and 20 μl supernatant was injected.

A Thermo-Finnigan Surveyor instrument (Thermo Electron, Rodano, Italy), equipped with autosampler and PDA-UV 6,000 LP detector, was used. Mass spectrometry analyses were performed using a LCQ Deca XP Plus spectrometer, with electrospray interface and ion trap as mass analyzer.

The chromatographic separations were run on a Varian Polaris C18-A column (150 $\times$ 2 mm, particle size 3 μm) (Varian, Leinì, Italy), with a flow rate of 200 μl/min. Gradient mobile phase composition was adopted: 95/5 to 0/100 vol/vol 5 mmol/l heptafluorobutanoic acid in water/methanol for 13 min.

The liquid chromatography 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; Claind, Lenno, Italy). The tuning parameters adopted for the electrospray ionization source were as follows: source current 80.00 μA, capillary voltage 3.00 V, and tube lens offset 15 V; and for ions optics, multipole one offset -5.25 V, intermultipole lens voltage -16.00 V, and multipole two offset -9.00 V. Mass spectra were collected in tandem mass spectrometry (MS) mode: MS^2 of (+) 379 m/z with 33% capillary electrophoresis in the range 100–400 m/z.

Oxidative biochemical parameters

Reduced glutathione (GSH), hydroxynonenal (HNE), and reactive oxygen species (ROS) levels were evaluated as reported elsewhere (12). For GSH levels, a mixture was directly prepared in a cuvette: 2.25 ml of 0.1 mol/l K-phosphate buffer, pH 8.0; 0.2 ml of the sample (plasma or PBMCs cytosol fractions); and 25 μl of Ellman's reagent (10 mmol/l dithionitrobenzoic acid in methanol). After 1 min, the assay absorbance was measured at 412 nm and the GSH concentration calculated by comparison with a standard curve.

HNE was determined on plasma or PBMC cytosol fractions. An aliquot of cy-

tosol (0.5 ml) was added to an equal volume of acetonitrile:acetic acid (96:4 vol:vol). After centrifugation, the supernatant was injected into a high-performance liquid chromatography (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.

ROS was measured in plasma or PBMC cytosol fractions using probe 2′,7′-dichlorofluorescin diacetate (DCFH-DA). DCFH-DA is a stable, nonfluorescent molecule that readily crosses the cell membrane and is hydrolyzed by intracellular esterases to the nonfluorescent 2′,7′-dichlorofluorescin (DCFH), which is rapidly oxidized, in the presence of peroxides, to highly fluorescent 2′,7′-dichlorofluorescein (DCF); DCF is measured fluorimetrically.

α-Tocopherol (vitamin E) was assayed using the method described by Burton et al. (13)—after extraction of 0.5 ml plasma with 1 ml n-heptane and centrifugation, the heptane phase was collected for HPLC analysis. A Supercosil-LC-Si column (25 cm $\times$ 4.6 mm; Supelco, Bellefonte, 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.

TNF-α and TNF receptors I and II

Serum levels

Serum levels of TNF-α, its soluble receptors TNF-RI (sTNF-RI) and TNF-RII (sTNF-RII) were measured using enzyme-linked immonosorbent assay tests (for TNF-α: Immunotech, Marseille, France; for sTNF-RI and sTNF-RII: Quantikine HS, R&D Systems, Minneapolis, MN) following the manufacturers' instructions.

Real-time RT-PCR

Total RNA was extracted from PBMCs using TRIzol Reagent (Invitrogen, Groningen, the Netherlands), as reported elsewhere (12). DNase was added to remove remaining genomic DNA. RNA was reverse transcribed using the iScript cDNA Synthesis Kit (BioRad Laboratories).

Primers were designed using the Beacon 5 program. Primers used were as follows: for TNF-α, forward 5′-CGC CAC CAC GCT CTT CTG C, reverse 5′-GGG

CTA CAG GCT TGT CAC TCG; for TNF-RI, forward 5′-CTG CCA GGA GAA ACA GAA CAC C, reverse 5′-GCG TCC TCA GTG CCC TTA ACA TTC; for TNF-RII: forward 5′-CGG TGT GGG CTG TGT CGT AG, reverse 5′- GAG GCT GCG GCT GTG GAG; and for β-actin, forward 5′-GCG AGA AGA TGA CCC AGA TC, reverse 5′-GGA TAG CAC AGC CTG GAT AG.

Real-time PCR was performed using a BioRad iQ iCycler Detection System with SYBR green fluorophore. Reactions were run in a total volume of 25 μl including 12.5 μl IQ SYBR Green Supermix (BioRad Laboratories), 1 μl of each primer at 10 μmol/l concentration, and 5 μl of the reverse-transcribed cDNA template. The protocol is as follows: denaturation (95°C for 5 min) and amplification repeated 40 times (95°C for 15 s, 60°C for 1 min). A melt curve analysis was performed to ensure a single amplified product for every reaction. All reactions were carried out in at least triplicate. Analysis of relative gene expression was performed using Gene Expression Macro software (BioRad Laboratories).

Statistical analysis
Results are presented as means ± SE. Differences between means were analyzed for significance using the Student's *t* test. A *P* value <0.05 was considered significant.

RESULTS — Oxidative stress was significantly higher in diabetic patients than control subjects, as demonstrated by higher levels of ROS and HNE and reduced levels of GSH and vitamin E (Table 1). Pentosidine levels, as well as sTNF-RI and sTNF-RII, were also higher in diabetic patients than in control subjects, whereas serum TNF-α levels were within the normal range (Table 1).

Table 1—BMI and serum concentrations of glucose, ROS, GSH, vitamin E, HNE, pentosidine, and cytokines in the study group at baseline

	Control subjects	Diabetic patients	*P*
n	20	20	
BMI (kg/m^2)	23.50 ± 1.12	25.50 ± 1.14	NS
Glucose (mg/ml)	78.00 ± 4.20	131.00 ± 7.70	<0.001
ROS (UF/ml)	111.9 ± 5.2	353.3 ± 42.3	<0.001
GSH (μg/ml)	91.6 ± 7.4	56.9 ± 9.0	<0.001
Vitamin E (μmol/l)	126.2 ± 8.5	65.8 ± 5.3	<0.001
HNE (μmol/l)	1.65 ± 0.21	5.15 ± 0.81	<0.05
Pentosidine (μmol/l)	0.75 ± 0.06	2.44 ± 0.18	<0.001
TNF-α (pg/ml)	2.34 ± 0.50	3.91 ± 1.00	NS
sTNF-RI (pg/ml)	1,039 ± 94	1,584 ± 100	<0.001
sTNF-RII (pg/ml)	1,792 ± 147	3,136 ± 291	<0.001

Data are means ± SE. Statistical analysis was performed using the unpaired data Student's *t* test.

Effects of DHEA on BMI and glycemic control
DHEA treatment had no effect on patients' BMI. Basal glucose concentration, A1C level, and HOMA index were also unaffected (Table 2).

Effects of DHEA on oxidative state
After DHEA treatment, DHEA and DHEAS levels were significantly increased (*P* < 0.05, Table 3). Oxidative stress parameters were significantly modified by DHEA treatment, both in plasma and in PBMCs. Plasma levels of ROS dropped by 53% (Table 3 and Fig. 1*A*). Likewise, a 47% reduction of plasma HNE was observed after DHEA treatment (Table 3 and Fig. 1*A*), whereas plasma levels of the nonenzymatic antioxidants GSH and vitamin E increased by 38 and 76%, respectively (Table 3 and Fig. 1*A*). The same trends were found in PBMCs (Table 3 and Fig. 1*B*). No correlation was found between DHEA or DHEAS levels and both oxidative and antioxidant parameters either before or after DHEA treatment.

Effects of DHEA on pentosidine levels
DHEA treatment markedly decreased pentosidine plasma concentration in diabetic patients (−50%; Table 3 and Fig. 1*A*).

Effects of DHEA on TNF-α and TNF-α receptors
After DHEA treatment, no modification of serum TNF-α and sTNF-RI was observed (Table 3), whereas in PBMCs mRNA expression was reduced by 33 and 29%, respectively (Fig. 1*C*). On the contrary, serum sTNF-RII levels were reduced after DHEA treatment (Table 3), without any modification in mRNA expression (Fig. 1*C*).

All the above parameters were unchanged in diabetic patients given placebo (data not shown).

Table 2—Characteristics of patients at baseline and after DHEA treatment (n = 10) or placebo administration (n = 10)

	DHEA (50 mg/dl)		Placebo	
	Baseline	12 weeks	Baseline	12 weeks
Age (years)	63.9 ± 11.1		58.0 ± 16.77	
BMI (kg/m^2)	25.7 ± 1.1	25.4 ± 1.4	25.7 ± 1.5	25.1 ± 1.4
Systolic blood pressure (mmHg)	127.7 ± 4.6	127.7 ± 3.0	137.0 ± 2.8	132.0 ± 2.7
Plasma glucose (mmol/l)	7.32 ± 0.37	7.40 ± 0.52	6.63 ± 0.81	6.36 ± 0.71
Creatinine (μmol/l)	78.7 ± 3.8	84.9 ± 5.9	81.3 ± 3.5	82.2 ± 2.9
LDL cholesterol (mmol/l)	3.00 ± 0.22	3.17 ± 0.27	3.82 ± 0.14	3.53 ± 0.31
A1C (%)	6.5 ± 0.2	6.8 ± 0.3	6.4 ± 0.5	6.7 ± 0.6
HOMA index	11.8 ± 1.9	12.7 ± 2.2	12.4 ± 2.0	11.3 ± 2.8

Data are means ± SE.

Table 3—*Effects of DHEA treatment on DHEA, DHEAS, oxidative and antioxidant parameters, and TNF-α/TNF-α receptor system in plasma and/or PBMCs*

| | DHEA (50 mg/die) | | | | | |
| | Plasma | | | PBMCs | | |
	Baseline	12 weeks	P	Baseline	12 weeks	P
ROS (UF/ml)	372.3 ± 66.6	183.0 ± 12.1	<0.05	307.4 ± 40.7	186.7 ± 37.1	<0.01
HNE (μmol/l)	6.40 ± 0.81	3.17 ± 0.42	<0.001	5.09 ± 0.27	2.60 ± 0.41	<0.001
GSH (μg/ml)	60.7 ± 2.0	85.7 ± 2.4	<0.01	314.4 ± 53.7	534.1 ± 73.8	<0.01
DHEA (nmol/l)	24.5 ± 2.7	32.8 ± 2.8	<0.05	ND	ND	
DHEAS (μmol/l)	1.7 ± 0.2	2.2 ± 0.1	<0.05	ND	ND	
Vitamin E (μmol/l)	68.3 ± 5.4	120.3 ± 10.9	<0.01	ND	ND	
Pentosidine (μmol/l)	2.31 ± 0.13	1.31 ± 0.06	<0.001	ND	ND	
TNF-α (pg/ml)	3.10 ± 1.40	6.88 ± 1.62	NS	ND	ND	
TNF-RI (pg/ml)	1602 ± 115	1591 ± 112	NS	ND	ND	
TNF-RII (pg/ml)	3,440 ± 365	2,829 ± 471	<0.01	ND	ND	

Data are means ± SE, $n = 10$. Statistical analysis was performed using the paired data Student's t test.

CONCLUSIONS — Type 2 diabetic patients with good glycemic control and no evidence of chronic diabetes complications, such as those enrolled in this study, show a redox imbalance characterized by increased production of highly reactive oxygen species and lower-than-normal antioxidant potential. We show here that DHEA treatment counteracts this oxidative imbalance—after 12 weeks, the concentrations of ROS and HNE were greatly reduced in both plasma and cytosol of PBMCs, whereas levels of the nonenzymatic antioxidants GSH and vitamin E were increased. These results are in agreement with the multi-targeted antioxidant effect of DHEA previously reported by our group (9,10,12). We could not find any correlation between serum DHEA or DHEAS levels and both the oxidative and the antioxidant parameters either before or after DHEA treatment. This result is not surprising, since DHEA is not a scavenger compound acting in a stoichiometric manner and exerts its antioxidants effects in a complex and non–completely defined way (10,12).

Moreover, DHEA treatment significantly reduced the plasma concentration of pentosidine in these patients, in line with its effects on AGEs and AGE receptors, which have been reported in experimental diabetes (12). AGEs, whose production is triggered by oxidative stress, are clearly implicated in the development and progression of chronic diabetes complications (3,4). Among AGEs, pentosidine is a well-characterized compound and is used as a marker of AGE biogenesis (14); it is considered a good predictor for the development of micro-

vascular complications in diabetic patients (15). Interestingly, DHEA reduces pentosidine concentration in type 2 diabetic patients without any influence on glycemic control, strongly suggesting that this effect can involve its ability to improve redox balance.

Hyperglycemia-induced oxidative stress may, either directly or through the AGE/AGE receptor interaction, serve as a key activator of upstream kinases, leading to an increase in the plasma inflammatory cytokine concentrations that is thought to be involved in the progression of chronic diabetes complications (6), as well as in the development of insulin resistance (16).

Compared with control subjects, diabetic patients showed higher levels of sTNF-RI and sTNF-RII, whereas TNF-α plasma concentrations were similar. After binding of TNF-α to its receptors, cleavage of the extracellular parts elicits the soluble forms, known as sTNF-RI and sTNF-RII, which are thought to reflect the degree of TNF system activation (17). TNF-α is not a very stable protein, and its serum level may not adequately reflect its activity. On the contrary, serum concentration of soluble TNF-α receptors have been shown to be increased in diabetic patients (18) and the level of sTNF-RII has been considered the best predictor of TNF-α system activation, as well as a marker of insulin resistance (19). DHEA treatment reduced the serum concentration of sTNF-RII and mRNA expression of TNF-α and TNF-RI in PBMCs. A reduction in mRNA expression of TNF-α indicates a downregulation of this system, which is reflected by a concomitant re-

duction in TNF-RI expression. As far as TNF-RII is concerned, our observation of a reduction in its serum levels without any change in its mRNA expression implies reduced shedding of the protein, suggesting improved insulin sensitivity after DHEA treatment. A relationship between TNF-α system and insulin resistance has indeed been reported (20), as has a negative correlation between TNF-RII shedding and insulin sensitivity (21). Moreover, ROS, which are markedly reduced by DHEA treatment, have recently been reported to play a causal role in insulin resistance (16). In accordance with previous observations (22), the present data suggest that DHEA treatment might influence insulin sensitivity in type 2 diabetic patients, through its effects on the TNF-α system, despite the absence of HOMA index modifications that we observed.

In conclusion, these data, together with the experimental data from rodents, suggest that DHEA treatment might prevent many of the events that lead to cellular damage induced by hyperglycemia, thus counteracting the onset and/or progression of chronic complications in type 2 diabetic patients. Of interest, this result was obtained in the absence of any improvement of glycemic control. Present management of hyperglycemia is based upon the assumption that the best way to reduce the risk of diabetes complications is to achieve optimal glycemic control. However, it should be pointed out that patients receiving intensive therapy designed to achieve glycemic control still develop diabetes complications, even though their prevalence is reduced (23).
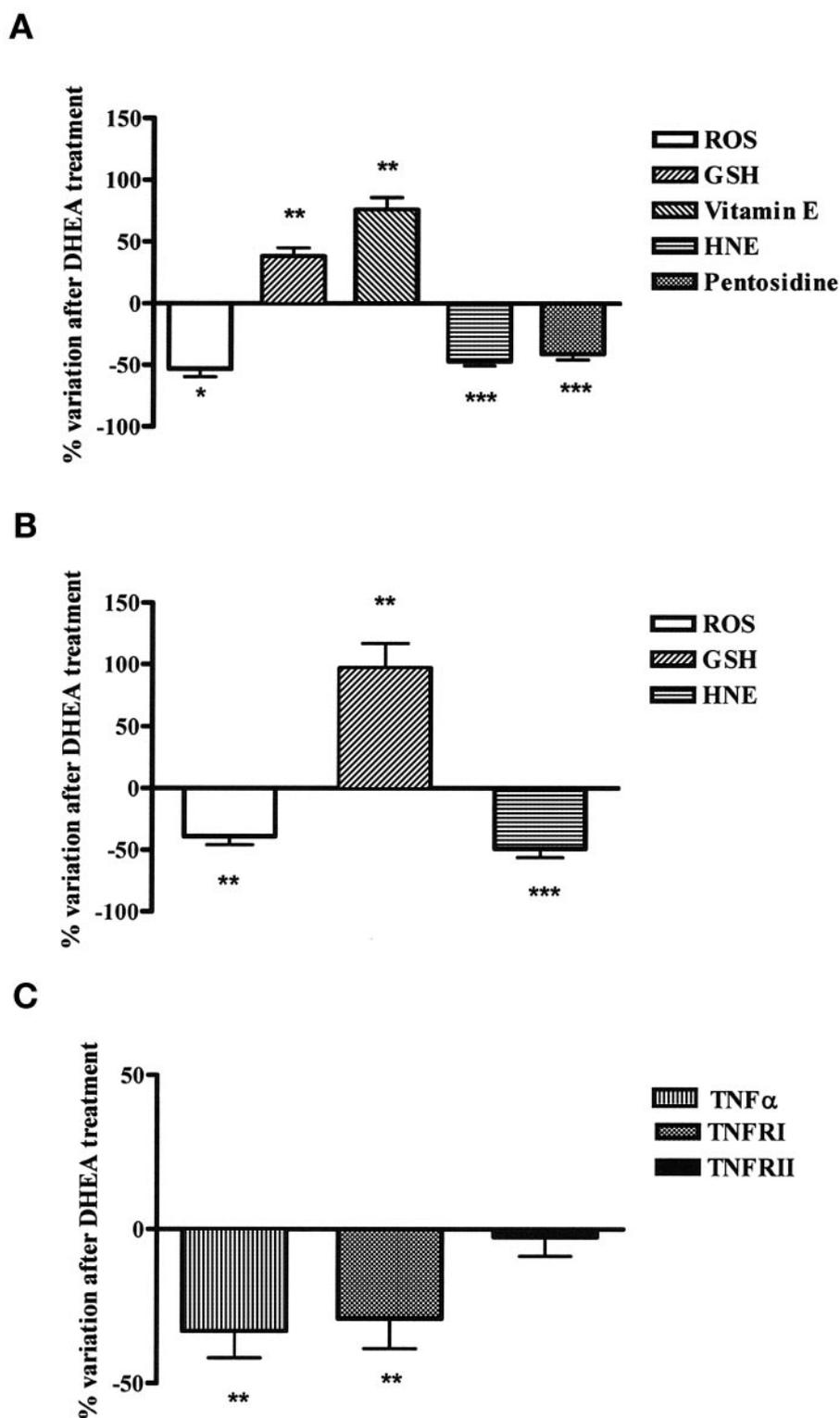
Figure 1—*Effects of DHEA on variations of oxidative stress parameters, TNF-α, and TNF-α receptors. A: Changes in levels of ROS, GSH, vitamin E, HNE, and pentosidine in plasma of diabetic patients treated with DHEA for 12 weeks. B: Changes in levels of ROS, GSH, and HNE in PBMCs of diabetic patients treated with DHEA for 12 weeks. C: Changes in mRNA expression of TNF-α, TNF-RI, and TNF-RII in PBMCs of diabetic patients treated with DHEA for 12 weeks. Variation is expressed as percentage with respect to levels at baseline. Significance vs. baseline: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.*

If hyperglycemia cannot be effectively prevented, the only way to impede diabetes complications will be to interrupt the pathways that lead from hyperglycemia to target organ damage. AGEs clearly represent one such pathway.

A similar preventive activity against hyperglycemia-induced oxidative stress has been postulated for drugs such as statins, ACE inhibitors, angiotensin II type 1 receptor blockers, calcium channel blockers, and thiazolidinediones, and their clinical use to prevent chronic complications in diabetic patients has been suggested (24). Compared with these drugs, DHEA has the advantage of being a physiologic steroid without side effects at the dosage used in this study. The usefulness of this novel approach to protect diabetic patients against tissue damage appears to be worth further exploration through multicenter clinical trials.

References
1. Baynes JW: Role of oxidative stress in development of complications in diabetes. *Diabetes* 40:405–412, 1991
2. Nishikawa T, Edelstein D, Du XL, Yamagishi S, Matsumura T, Kaneda Y, Yorek MA, Beebe D, Oates PJ, Hammes HP, Giardini I, Brownlee M: Normalizing mitochondrial superoxide production blocks three pathways of hyperglycaemic damage. *Nature* 404:787–790, 2000
3. Brownlee M: Biochemistry and molecular cell biology of diabetic complications. *Nature* 414:813–820, 2001
4. Vlassara H: The AG: E-receptor in the pathogenesis of diabetic complications. *Diabete Metab Res Rev* 17:436–443, 2001
5. Forbes JM, Yee LT, Thallas V, Lassila M, Candido R, Jandeleit-Dahm KA, Thomas MC, Burns WC, Deemer EK, Thorpe SM, Cooper ME, Allen TJ: Advanced glycation end product interventions reduce diabetes-accelerated atherosclerosis. *Diabetes* 53:1813–1823, 2004
6. Esposito K, Nappo F, Marfella R, Giugliano G, Giugliano F, Ciotola M, Quagliaro L, Ceriello A, Giugliano D: Inflammatory cytokine concentrations are acutely increased by hyperglycemia in humans: Role of oxidative stress. *Circulation* 106: 2067–2072, 2002
7. Lonn E, Yusuf S, Hoogwerf B, Pogue J, Yi Q, Zinman B, Bosch J, Gagenais G, Mann JFE, Gerstein HC: Effects of vitamin E on cardiovascular and microvascular out-

comes in high-risk patients with diabetes. *Diabetes Care* 25:1919–1927, 2002

8. Brignardello E, Beltramo E:, Molinatti PA, Aragno M, Gatto V, Tamagno E, Danni O, Porta M, Boccuzzi G: Dehydroepiandrosterone protects bovine retinal capillary pericytes against glucose toxicity. *J Endocrinol* 158:21–26, 1998

9. Aragno M, Parola S, Brignardello E, Mauro A, Tamagno E, Manti R, Danni O, Boccuzzi G: Dehydroepiandrosterone prevents oxidative injury induced by transient ischemia/reperfusion in the brain of diabetic rats. *Diabetes* 49:1924–1931, 2000

10. Aragno M, Mastrocola R, Medana C, Restivo F, Catalano MG, Pons N, Danni O, Boccuzzi G: Up-regulation of advanced glycated products receptors in the brain of diabetic rats is prevented by antioxidant treatment. *Endocrinology* 146:561–5567, 2005

11. Matthews DR, Hosker JP, Rudenski AS, Naylor BA, Treacher DF, Turner RC: Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man. *Diabetologia* 28:412–419, 1985

12. Aragno M, Mastrocola R, Medana C, Catalano MG, Vercellinatto I, Danni O, Boccuzzi G: Oxidative stress-dependent impairment of cardiac-specific transcription factors in experimental diabetes. *Endocrinology* 147:5967–5974, 2006

13. Burton GW, Traber MG, Acuff RV, Walters DN, Kayden H, Hughes L, Ingold KU: Human plasma and tissue alpha-to-copherol concentrations in response to supplementation with deuterated natural and synthetic vitamin E. *Am J Clin Nutr* 67:669–684, 1998

14. Raj DSC, Choudhury D, Welbourne TC, Levi M: Advanced glycation end products: a nephrologist's perspective. *Am J Kidney Dis* 35:365–380, 2000

15. Monnier VM, Bautista O, Kenny D, Sell DR, Fogarty J, Dahms W, Cleary PA, Lachin J, Genuth S, the DCCT Skin Collagen Ancillary Study Group: Skin collagen glycation, glycoxidation, and crosslinking are lower in subjects with long-term intensive versus conventional therapy of type 1 diabetes: relevance of glycated collagen products versus HbA1c as markers of diabetic complications. *Diabetes* 48:870–880, 1999

16. Houstis N, Rosen ED, Lander ES: Reactive oxygen species have a causal role in multiple forms of insulin resistance. *Nature* 440:944–948, 2006

17. Nophar Y, Kemper O, Brakebusch C, Englemann H, Zwang R, Aderka D, Holtmann H, Wallach D: Soluble forms of tumor necrosis factor receptors (TNF-Rs): the cDNA for the type I TNF-R, cloned using amino acid sequence data of its soluble form, encodes both the cell surface and a soluble form of the receptor. *EMBO J* 9:3269–3278, 1990

18. Fernandez-Real JM, Brich M, Ricart W, Casamitjana R, Gutierrez C, Vendrell J, Richart C: Plasma levels of the soluble fraction of tumor necrosis factor receptor 2 and insulin resistance. *Diabetes* 47:1757–1762, 1998

19. Hotamisligil GS, Arner P, Atkinson RL, Spiegelman BM: Differential regulation of the p80 tumor necrosis factor receptor in human obesity and insulin resistance. *Diabetes* 46:451–455, 1997

20. Winkler G, Lakatos P, Salamon F, Nagy Z, Speer G, Kovacs M, Harmos G, Dworak O, Cseh K: Elevated serum TNF-alpha level as a link between endothelial dysfunction and insulin resistance in normotensive obese patients. *Diabet Med* 16:207–211, 1999

21. Fernandez-Real JM, Lainez B, Vendrell J, Rigla M, Castro A, Penarroja G, Broch M, Perez A, Richart C, Engel P, Ricart W: Shedding of TNF-alpha receptors, blood pressure, and insulin sensitivity in type 2 diabetes mellitus. *Am J Physiol Endocrinol Metab* 282:E952–E959, 2002

22. Kawano H, Yasue H, Kitagawa A, Hirai N, Yoshida T, Soejima H, Miyamoto S, Nakano M, Ogawa H: Dehydroepiandrosterone supplementation improves endothelial function and insulin sensitivity in men. *J Clin Endocrinol Metab* 88:3190–3195, 2003

23. Soro-Paavonen A, Forbes JM: Novel therapeutics for diabetic micro- and macrovascular complications. *Curr Med Chem* 13:1777–1788, 2006

24. Ceriello A: Controlling oxidative stress as a novel molecular approach to protecting the vascular wall in diabetes. *Curr Opin Lipidol* 17:510–518, 2006