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ORIGINAL ARTICLE

Impaired HPA axis function in diabetes involves adrenal apoptosis and phagocytosis

Esteban M. Repetto[1,2] · Morena Wiszniewski[2] · Ana L. Bonelli[2] · Carolina V. Vecino[2] · Camila Martinez Calejman[2] · Pablo Arias[3] · Cora B. Cymeryng[2,4]

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Abstract

Purpose The aim of the present study was to analyze the involvement of oxidative stress and inflammation in the modulation of glucocorticoid production in the adrenal cortex of diabetic rats.

Methods Male Wistar rats were treated with or without streptozotocin (STZ, an insulinopenic model of diabetes) and either α-lipoic (90 mg/kg ip.), α-tocopherol (200 mg/kg po.) or with STZ and supplemented with insulin (STZ + INS: 2.5U/day) for 4 weeks. Oxidative/nitrosative stress parameters and antioxidant enzymes were determined in adrenocortical tissues. Apoptosis and macrophage activation were evaluated by immunohistochemistry (TUNEL and ED1^{+}). Basal and ACTH-stimulated corticosterone production were assessed by RIA and plasma ACTH levels were determined by an immunometric assay.

Results Diabetic rats showed a diminished response to exogenous ACTH stimulation along with higher basal corticosterone and lower plasma ACTH levels. In the adrenal cortex we determined an increase in the levels of lipoperoxides, S-nitrosothiols, nitric oxide synthase activity and nitro-tyrosine modified proteins while catalase activity and heme oxygenase-1 expression levels were also elevated. Antioxidant treatments were effective in the prevention of these effects, and in the increase in the number of apoptotic and phagocytic (ED1^{+}) cells detected in diabetic rats. No changes were observed in the STZ + INS group.

Conclusions Generation of oxidative/nitrosative stress in the adrenal cortex of diabetic rats leads to the induction of apoptosis and the activation of adrenocortical macrophages and is associated with an elevated basal corticosteronemia and the loss of the functional capacity of the gland.

Keywords Diabetes · Oxidative stress · Apoptosis · HPA dysfuntion · α-tocopherol · α-lipoic acid

Introduction

Type 1 diabetes is characterized by hyperglycemia, hypertriglyceridemia, and an increased in circulating non-esterified fatty acids (NEFAs). This pronounced imbalance in nutrient availability to the cells is associated with metabolic derangements usually leading to an increased production of reactive oxygen and/or nitrogen species, as demonstrated both in patients and in animal models of this disease [1, 2]. Accordingly, several studies have suggested that development of most of the long-term complications associated with diabetes entails an increase in the generation of oxidative and nitrosative stress [3–5]. Based on these evidences, a variety of antioxidant molecules have been tested for their ability to prevent the progression of diabetic complications with inconclusive results [6–8].

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✉ Esteban M. Repetto
erepetto@fmed.uba.ar

[1] Departamento de Bioquímica Clínica, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Buenos Aires, Argentina

[2] Facultad de Medicina, Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Centro de Estudios Farmacológicos y Botánicos (CEFYBO), Laboratorio de Endocrinología Molecular (LEM), Universidad de Buenos Aires, Buenos Aires, Argentina

[3] Departamento de Fisiología, Facultad de Medicina, Universidad Nacional de Rosario, Rosario, Argentina

[4] Departamento de Bioquímica Humana, Facultad de Medicina, Universidad de Buenos Aires, Buenos Aires, Argentina

Several studies have demonstrated the beneficial effects of dietary supplementation with micronutrients with antioxidant properties, as α-lipoic (or thioctic) acid and α-tocopherol (vitamin E), as a complement to classical therapies in the prevention or treatment of diabetic complications [9, 10].

Alpha-lipoic acid is a dithio-compound involved in the generation of energy in the mitochondria as an essential cofactor, and based on its potent antioxidant properties it has been used in the prevention and therapy of a broad range of disorders. Both lipoic acid and its metabolite dihydrolipoic acid are readily absorbed from the diet and fulfill many of the requirements to be considered good antioxidants as they are potent scavengers of reactive oxygen and nitrogen species both in lipid and aqueous domains and have a metal chelating activity [11–14]. In addition, they are involved in the regeneration of other antioxidants, as vitamin E and C and reduced glutathione [11, 15].

As detailed in recent reviews, α-lipoic acid exerts a number of beneficial effects in managing diabetic complications [9, 16]. As an example, lipoic acid has been demonstrated to alleviate diabetic neuropathy [17–19], diabetic nephropathy [20, 21], and diabetic cardiomyopathy [22].

Alpha-tocopherol is a fat-soluble nutrient with potent antioxidant activity, and its presence in biological membranes is thought to represent a major defense system against free radical-mediated lipid peroxidation [23]. As a downside, this antioxidant molecule acts only within membranes or lipid domains, with little activity in aqueous phases [14].

The effectiveness of vitamin E in the prevention of diabetic complications has been analyzed in several trials. As examples, Khatami et al. demonstrated, in a randomized, double-blind, placebo-controlled trial, that supplementation with an oral high-dose of vitamin E for 12 weeks had favorable effects on biomarkers of kidney injury, inflammation, and oxidative stress [24]; Gupta et al. demonstrated that vitamin E ameliorates oxidative stress in patients with diabetes mellitus type 1 and improves the antioxidant defense system without modifying metabolic parameters [25] and Giannini et al. demonstrated that high-dose vitamin E supplementation reduces markers of oxidative stress and improves antioxidant defense in young patients with type 1 diabetes [26].

In a previous study we demonstrated the dysregulation of basal and ACTH-stimulated corticosterone production in rats treated with streptozotocin (STZ), an insulinopenic model of diabetes mellitus [27]. In these diabetic animals we determined significantly increased serum basal corticosterone levels and a lower response to exogenous ACTH, four weeks after the confirmation of hyperglycemia. Analysis of the adrenal cortex revealed the generation of oxidative/nitrosative stress in diabetic rats, as lipid peroxidation products, protein carbonyls, nitric oxide synthase (NOS) activity, nitrotyrosine modified proteins, and the expression levels of both endothelial nitric oxide synthase (NOS3) and neuronal NOS (NOS1) were all increased. We thus hypothesized that, deleterious effects attributed to the generation of oxidative/nitrosative stress in the adrenal cortex, as a consequence of the increased metabolic burden, are involved in the dysregulation of adrenocortical steroidogenesis in STZ-diabetic rats [27]. Present experiments were designed in order to test this hypothesis.

Materials and methods

Chemicals

Synthetic porcine corticotropin lyophilized was obtained from Laboratorios Elea (Buenos Aires, Argentina). STZ was from Sigma-Aldrich (St Louis, MO, USA). MMLV reverse transcriptase and DNAse (RNAse free) were from Promega (Madison, WI, USA). Taq DNA polymerase and Trizol reactive were from Invitrogen, Life Technologies (Gaithersburg, MD, USA). α-tocopherol was bought from Laboratorios Raymos (BA, Argentina) and α-lipoic acid was from Laboratorios Gador (BA, Argentina). Anti-corticosterone antibodies were kindly provided by Dr. A. Belanger (Laval University, Quebec, Canada). Anti HO1 was obtained from StressGen Biotechnologies Corp. (#SPA-896, Victoria, Canada). ED-1 and 3-nitrotyrosine antibodies were from Abcam (#Ab31630, Cambridge, UK) and Sigma-Aldrich Inc. (#N0409, St. Louis,MO, USA) respectively. Actin, caspase-3 p17 and peroxidase-conjugated horse anti-mouse-IgG antibodies were obtained from Santa Cruz Biotechnology (#sc-47778, #sc-373730, Dallas, TX, USA) and Cell Signaling Technology (#7076, Danvers, MA, USA), respectively. The secondary antibody conjugated with peroxidase goat anti-rabbit-IgG and the cation exchange resin 50W-X8 were from BioRad (Hercules, CA, USA). All the other reagents were of the highest quality available.

Animals and experimental procedures

Adult male Wistar rats (220–240 g body weight) were maintained at 21 ± 1 °C on a 12–12 h light-to-dark cycle. Diabetes mellitus was induced by STZ (40 mg/kg/d in 0.1 mol/L sodium citrate, pH 4.2, i.p., 3 doses/days). Control animals were treated with citrate buffer solution. During the first 48 h after STZ injection, the animals were administered 20% v/v sucrose to compensate for hypoglycemia resulting from massive insulin release from the pancreas. All animals received food and water ad libitum. Only thosec

animals from the STZ-group presenting glycaemia higher than 16.7 mmol/L were used in the following experiments. In the first group of experiments, animals were randomly distributed in two groups: control and STZ ($n = 5$) and animals were sacrificed 4 weeks after STZ treatment. In the second experiment, animals were assigned to the following groups: control, STZ, α-lipoic acid (90 mg/kg ip, every 48 h), α-tocopherol (200 mg/kg p.o., every 48 h), STZ-lipoic acid or STZ-tocopherol. A group of STZ-treated rats was inserted with a slow-release insulin implant (STZ with insulin treatment group = two linplants per rat releasing approximately 2.5 U insulin/day; Linshin® Canada Inc., Scarborough, Ontario, Canada). Antioxidant and insulin treatments were initiated immediately after the confirmation of STZ-induced hyperglycemia and continued for the duration of the experimental period.

An ACTH stimulatory test was performed during the 4th week after STZ treatment. Briefly, rats were injected with ACTH (7 IU/kg i.p.) and blood was collected 60 min after.

Animals were sacrificed four weeks after the confirmation of hyperglycemia during the morning hours (between 0900 and 1000 h), according to protocols approved by the Animal Care and Use Committee (CICUAL), Facultad de Medicina, Universidad de Buenos Aires and were conducted in agreement with the "Principles of laboratory animal care" (NIH publication no. 85-23, revised 1985). Trunk blood was collected and plasma and serum were obtained for ACTH and corticosterone determinations, respectively.

Biochemical and hormonal determinations

Corticosteronemia was determined by a radioimmunoassay as previously described [27]. The minimal detectable concentration was 1.0 nmol/L and the inter-assay and intra-assay coefficients of variation were 5.9 and 4.9%, respectively. Plasma ACTH levels were determined using Immulite 2000® (Siemens, Erlange, Germany). The minimal detectable concentration was 1.1 pmol/L and inter-assay and intra-assay coefficients of variation were 10 and 9.5%, respectively. Fasting serum glucose, total and HDL-cholesterol and triglyceride levels were assessed by enzymatic colorimetric commercial assays (Wiener Lab, Rosario, Argentina). HDL-cholesterol was determined in the supernatant after selective precipitation of other lipoproteins from serum with dextran sulfate-magnesium chloride (Wiener Lab, Rosario, Argentina). The non HDL-cholesterol fraction was calculated as total cholesterol levels minus HDL-cholesterol level.

Zona fasciculata tissue preparation

Adrenal glands were rapidly dissected and placed on a chilled plate. Zona fasciculata adrenal tissue was homogenized in 0.2 ml/gland of 10 mM HEPES (pH 7.4), 320 mM sucrose, 0.1 mM EDTA, 0.1 mM dithiothreitol and the following protease inhibitors: 2 µg/ml pepstatin, 10 µg/ml leupeptin, 25 µg/ml aprotinin, and 2 mM phenylmethylsulfonyl fluoride. The homogenate was centrifuged at 800×*g* for 10 min and then at 9000×*g* for 20 min, and the supernatant was used in the following determinations.

Nitric oxide synthase (NOS) activity

NOS activity was determined by monitoring the conversion of L-[^{3}H] arginine to L-[^{3}H] citrulline as described elsewhere [27]. NOS enzyme activity is indicated as picomoles of L-[^{3}H]-citrulline formed per mg protein/min.

Measurement of lipid peroxides and S-nitrosothiols (RSNO) levels

Thiobarbituric acid reactive species (TBARS) were determined as previously described [27]. RSNO levels were detected by a fluorometric reaction [28]. Briefly, the interaction of RSNOs with Hg^{+2} generates NO^+ and a fluorescent derivative is formed in the presence of 2,3 diaminonaphthalene at pH 7.4. The fluorescent adduct is quantified using excitation and emission wavelengths of 375 and 456 nm, respectively.

Antioxidant enzymes activity

Heme oxygenase (HO) activity was determined as described [29]. Catalase activity was measured according to published procedures [30] with minor modifications. Briefly, adrenal homogenates were diluted 1/10 in 50 mM sodium phosphate buffer pH 7.4 and 100 µl of this dilution were placed in a spectrophotometric cuvette. Reactions were initiated by the addition of 60 µl of 2.5 Vol H_2O_2 (223 mM) and the absorbance at 240 nm was monitored for 120 s. The activity, expressed as µM of H_2O_2 consumed per min per µg protein, was calculated from the slope of the corresponding curve, considering a molar absorptivity of 0.0394 $mM^{-1}cm^{-1}$ for H_2O_2.

RNA isolation and quantitative real time-PCR (qRT-PCR) assay

Total RNA was obtained from the rat adrenal cortex using Trizol® reagent according to the manufacturer's instructions and cDNA was synthesized as described [31]. Amplifications by real-time PCR were carried out in a Rotor-Gene 6000 Corbett Life Science Real Time Thermal Cycler (Corbett Research, Sydney, NSW, Australia) and quantified with the Rotor Gene 6000 Series Software (version 1.7

Build 40, Hilden, Germany). Primer oligonucleotide sequences used in this study were as follows: *Star* F: 5′-CT GCTAGACCAGCCCATGGAC-3′, R: 5′-TGATTTCCTT-GACATTTGGGTTCC-3′; *Mc2r* F: 5′-GATGCTGGTTT TTATCCTGTGTC-3′, R: 5′-ATGCCATTGACCTGGA AG AG-3′; *Cyp11a1* F: 5′-GGGTGGCCTATCACCAG-TAT-3′, R: 5′-CTGAGCTACACCTT CCAGCA-3′; *18S RNA* F: 5′-ACGGAAGGGCACCACCAGGA-3′, R: 5′-CACCACCAC CCACGGAATCG -3′.

Immunoblot analysis

Immunoblot analysis was performed as previously described [27]. Membranes were incubated overnight with heme oxygenase 1 (1:1000), 3-nitrotyrosine (1:2000) or actin (1:1000) antisera at 4 °C and a 1:20,000 dilution of a goat anti-rabbit IgG antibody horseradish peroxidase conjugate was used as a secondary antibody. Bands were detected by chemiluminescence.

Histological evaluation and immunohistochemical studies

Deeply anesthetized were intracardially perfused with saline, followed by a fixative solution (4% paraformaldehyde in phosphate buffer, pH 7.2). Adrenals were obtained and post-fixed for 24 h at 4 °C. After several washes, tissue samples were dehydrated, cleared in xylene and embedded in paraffin. Serial longitudinal sections 5 µm thick were obtained using a microtome (Leica, Leica Microsystems, Buenos Aires, Argentina). For histological analysis, adrenal sections were stained with hematoxylin and eosin (H&E). Antigen retrieval was performed by heating slices at 90 °C for 30 min in citrate buffer (pH 6.3). Adrenal sections were preincubated with 2% normal horse serum for 1 h, and then were incubated overnight at 4 °C with mouse anti-ED1 or anti-caspase-3 p17 (cleaved form) antibody. After several washes, donkey anti-mouse secondary antibody conjugated to Alexa 488 was added, and sections were incubated for additional 2 h at room temperature. Regularly, primary antibodies were omitted in some sections to confirm specificity. Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI), mounted using anti-fade medium (Vectashield, Vector Laboratories, CA, USA), and observed under a fluorescence microscope (Nikon Eclipse Ti-E PFS), connected to a digital camera (ANDOR Neo 5.5 sCMOS.) attached to a computer running image analysis software (Optimus, Media Cybernetics, Silver Spring, MD, USA). Comparative digital images from different samples were obtained using identical exposure time, brightness, and contrast settings. ED1 positive area was measured in adrenal sections and expressed as percentage of an identical total area. NIH ImageJ Software (National Institutes of Health, Bethesda, Maryland) was used to quantify the intensity.

TUNEL assay

Quantitative analysis of apoptosis was performed by fluorescein in situ Terminal deoxynucleotidyl transferase dUTP Nick- End Labeling (TUNEL) assay according to the manufacturer's instructions (EMD Millipore, Massachusetts). Nuclei were counterstained with DAPI. A negative control was generated by the omission of the TdT enzyme. Slices were observed under a fluorescence microscope (Nikon Eclipse Ti-E PFS, Tokyo, Japan).

To quantify the number of apoptotic cells, TUNEL positive cells were counted manually by assessing ten random fields per experimental condition. The apoptotic index was calculated as the percentage of nuclei stained TUNEL-positive divided by the total number of DAPI-stained nuclei in each section.

Statistical analysis

Data were tested for normality using the Kolmogorov-Smirnoff test. All values are expressed as mean ± SEM of n samples, at least three independent experiments. Differences between groups were analyzed by unpaired t-test or by factorial one-way ANOVA, as appropriate. When the ANOVA yielded significant differences ($P < 0.05$) post hoc comparisons (Tukey's test) were made to determine the statistical levels of difference between groups. All calculations were performed using GraphPad InStat version 3.06 for Windows (GraphPad Software, San Diego, CA, USA).

Results

Compared to controls, diabetic rats presented clearly elevated blood glucose as well as triglyceride levels as well as a significant reduction in body weight four weeks after the STZ injection (Table 1). No significant differences in total cholesterolemia were detected between groups (Table 1) but analysis of cholesterol-containing lipoproteins indicated a significant decrease in HDL-cholesterol levels (control: 1.16 ± 0.08, STZ: 0.92 ± 0.03 $P < 0.01$) and a concomitant increase in non-HDL-cholesterol levels (control: 0.63 ± 0.11, STZ: 0.92 ± 0.07 $P < 0.05$).

Although basal corticosteronemia was markedly elevated in the diabetic group (STZ), the functional capacity of adrenal glands, determined as the corticosterone response 60 min after exogenous ACTH stimulation, was significantly reduced in diabetic animals (Fig. 1a). Compared

Table 1 Body weight, biochemical, and morphometric parameters determined in control and diabetic rats (STZ) with and without antioxidant treatment and insulin supplemented diabetic rats (STZ + Insulin)

	Control	α-tocopherol	α-lipoic acid	STZ	STZ + α-tocopherol	STZ + α-lipoic acid	STZ + Insulin
n	12	6	6	12	9	9	6
Body weight (g)	421.5 ± 10.2	401.3 ± 14.3	412.4 ± 12.4	360.3 ± 13.1**	334.1 ± 8.1***	356.4 ± 14.3**	460.0 ± 9.5$^{\#\#}$
Glucose (mmol/L)	4.5 ± 0.2	5.3 ± 0.3	5.8 ± 0.2	28.0 ± 1.5***	27.9 ± 2.0***	33.5 ± 1.9***	4.8 ± 0.8$^{\#\#\#}$
Triglycerides (mmol/L)	1.32 ± 0.15	1.61 ± 0.11	0.98 ± 0.08	1.97 ± 0.15*	2.35 ± 0.25***	1.73 ± 0.17	1.20 ± 0.08$^{\#}$
Total cholesterol (mmol/L)	1.79 ± 0.11	1.91 ± 0.10	1.65 ± 0.08	1.84 ± 0.07	1.83 ± 0.05	1.67 ± 0.06	1.55 ± 0.12
Adrenal weight (mg)	29.7 ± 1.7	26.4 ± 1.5	30.3 ± 2.1	34.3 ± 2.3	36.6 ± 1.2	35.7 ± 1.7	31.6 ± 3.0
Relative adrenal weight (mg/Kg)	69.2 ± 3.6	69.2 ± 1.6	69.4 ± 6.7	93.9 ± 5.3**	97.9 ± 3.7**	108.4 ± 3.3***	68.6 ± 6.4$^{\#}$

Values are presented as mean ± SEM; *n* indicates the number of rats used in each group; $^{***}p < 0.001$, $^{**}p < 0.01$, and $^{*}p < 0.05$ vs. control and, $^{\#\#\#}p < 0.001$, $^{\#\#}p < 0.01$, and $^{\#}p < 0.05$ vs. STZ (ANOVA followed by Tukey's test)

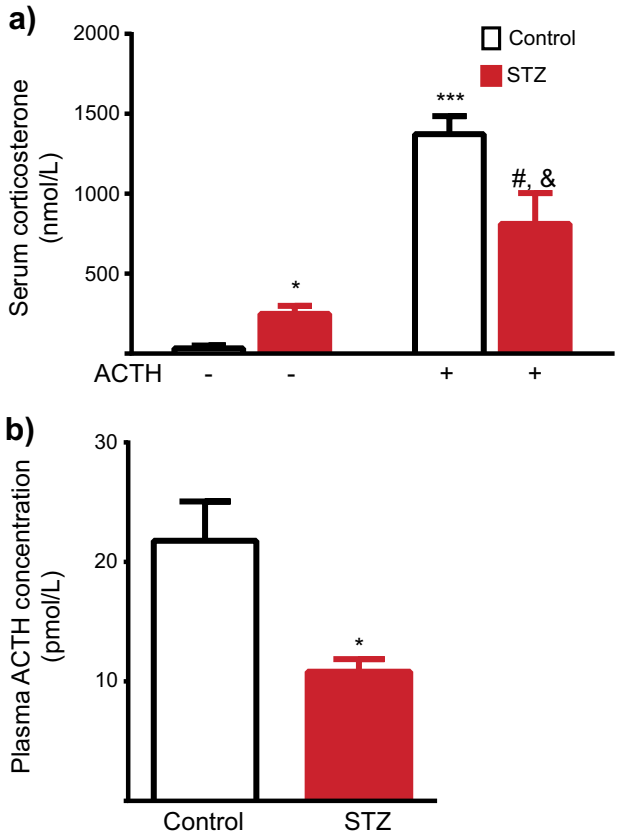

Fig. 1 A dysfunction of the hypothalamic-pituitary-adrenal axis is already evident four weeks after STZ-treatment. **a** Serum corticosterone levels were determined by radioimmunoassay in samples obtained before and 60 min after ACTH stimulation (7 IU/kg, ip) in both Control (white bars) and STZ-induced diabetic rats (red bars). **b** Basal plasma ACTH levels were determined by a chemiluminiscent assay in both groups. Data is shown as mean ± SEM, $n = 5$ per group. $^{*}p < 0.05$ and $^{***}p < 0.001$ vs. control, $^{\#}p < 0.05$ vs. STZ, $^{\&}p < 0.05$ vs. Control + ACTH (ANOVA followed by Tukey's test)

to controls, plasma ACTH levels were lower in STZ-induced diabetic rats (Fig. 1b).

As depicted in Fig. 2, we detected increased levels of lipid peroxidation products, NOS activity, S-nitrosothiols, and nitrotyrosine modified proteins in adrenocortical tissues obtained from diabetic rats (Fig. 2a–d). Adrenocortical antioxidant defense enzymes were analyzed in order to determine if an antioxidant defense response develops in insulin-deprived rats. Figure 2e, f show that the activities of both catalase and heme oxygenase were significantly increased in the STZ-group, while no significant differences were observed in the activities of superoxide dismutase or glutathione peroxidase at this time point (data not shown). These results prompted us to assess the effect of two well-known antioxidant molecules in biochemical and hormonal parameters.

Antioxidant treatment (α-lipoic acid or α-tocopherol) had no effect on systemic metabolic parameters (blood glucose, triglycerides, total cholesterol,) or adrenal and body weight in either control or diabetic rats (Table 1). As a control for off-target effects of STZ, the normalizing effect of insulin therapy on these parameters in STZ-induced diabetic rats is also shown (Table 1).

In spite of the lack of effect on systemic parameters, both antioxidants exerted a significant prevention of diabetes-dependent induction of lipoperoxide generation and in the increase in NOS activity in the adrenal cortex (Fig. 3a, b). Changes in the activity of antioxidant enzymes were also prevented by both compounds (Fig. 3c, d).

Histological evaluation of adrenocortical tissues by H&E staining indicated no significant differences between groups 4 weeks after the induction of diabetes (Fig. 4a, supplementary figure 1). However, immunostaining with ED1, a marker of activated macrophages (active phagocytosis) showed an increased signal in sections obtained from diabetic rats. Both antioxidant treatments (α-lipoic acid or α-tocopherol) prevented these changes (Fig. 4a, b).

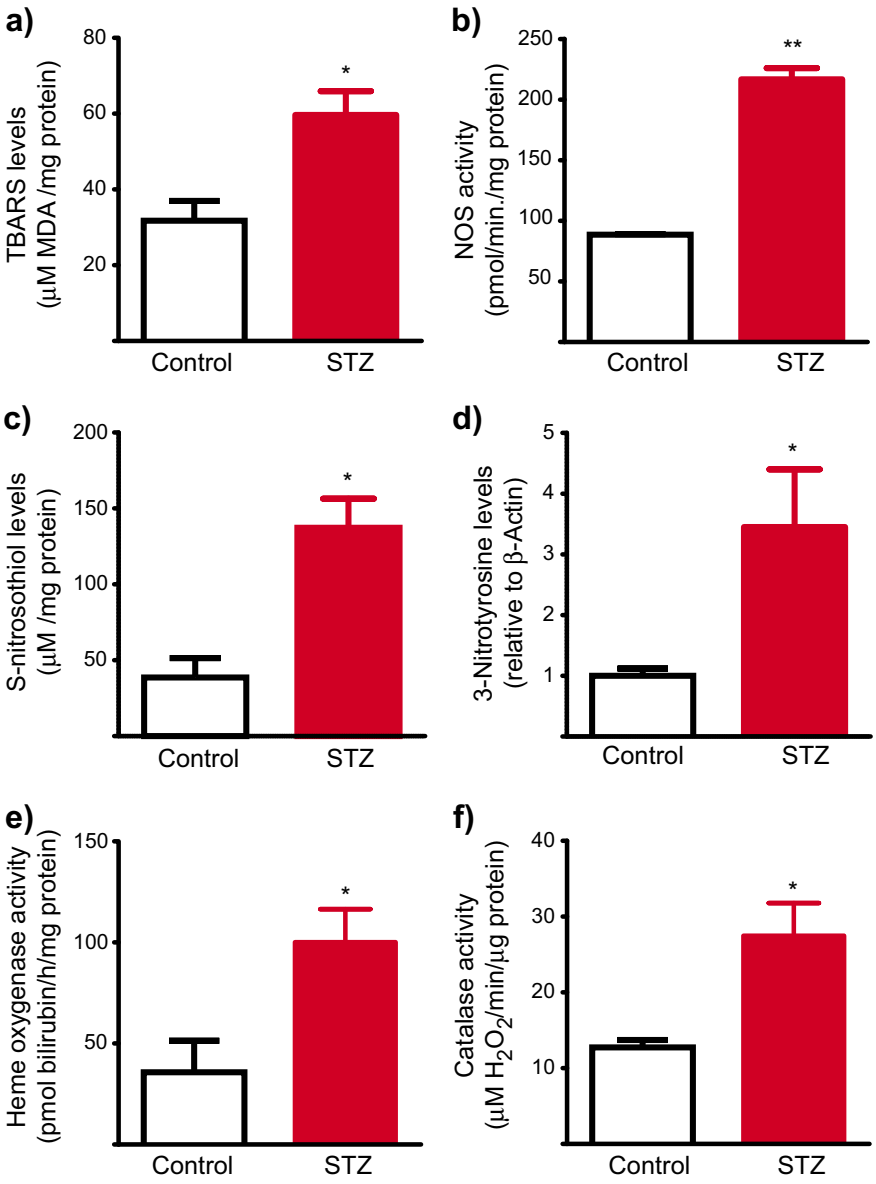

Fig. 2 Generation of oxidative and nitrosative/ stress and induction of antioxidant enzymes in the adrenal cortex of diabetic rats. **a** Lipid peroxidation levels, as thiobarbituric acid reactive substances (TBARS) with malondialdehyde (MDA) as a standard. **b** Nitric oxide synthase activity, **c** S-nitrosothiol levels, **d** 3-nitrotyrosine modified protein levels and enzimatic activities of **e** Heme oxygenase and **f** Catalase. Values are expressed as mean ± SEM $n = 5$ per group. $*p < 0.05$ and $**p < 0.01$ vs. Control (unpaired *t*-test)

Considering that adrenal dysfunction could be due to an increase in the number of cells irreversibly damaged as a consequence of the induction diabetes-related processes, we also evaluated apoptotic cells in sections from all groups. Our results showed a significant increase in the number of TUNEL positive cells quantified in adrenal sections from diabetic animals. Antioxidant treatment also markedly attenuated the increase in this parameter (Fig. 4a, c).

Additionally, as a control, analysis of tissues obtained from diabetic animals supplemented with insulin (STZ + INS), indicated that hormonal treatment blocked the induction of apoptosis (caspase-3 p17, a cleaved active form) and phagocytosis (ED1 staining) in the adrenal cortex of STZ-induced diabetic rats (supplementary Figure 1).

Finally, assessment of adrenocortical function in antioxidant-treated animals indicated that both molecules normalized basal and ACTH-stimulated corticosterone output with no significant effect on control animals (Fig. 5a, b). Although compared to controls, a significant down-regulation of adrenocortical *Mc2r* and an increase in *Star* mRNA levels were detected in the diabetic group, no differences were observed in these parameters in diabetic rats treated with antioxidants (Fig. 5c). Plasma ACTH levels and HDL and non-HDL cholesterol serum levels were not affected by antioxidant treatment (data not shown).

Discussion

Glucocorticoids participate in the control of whole-body homeostasis modulating the organism's response to stress. It is posited that baseline permissive levels of

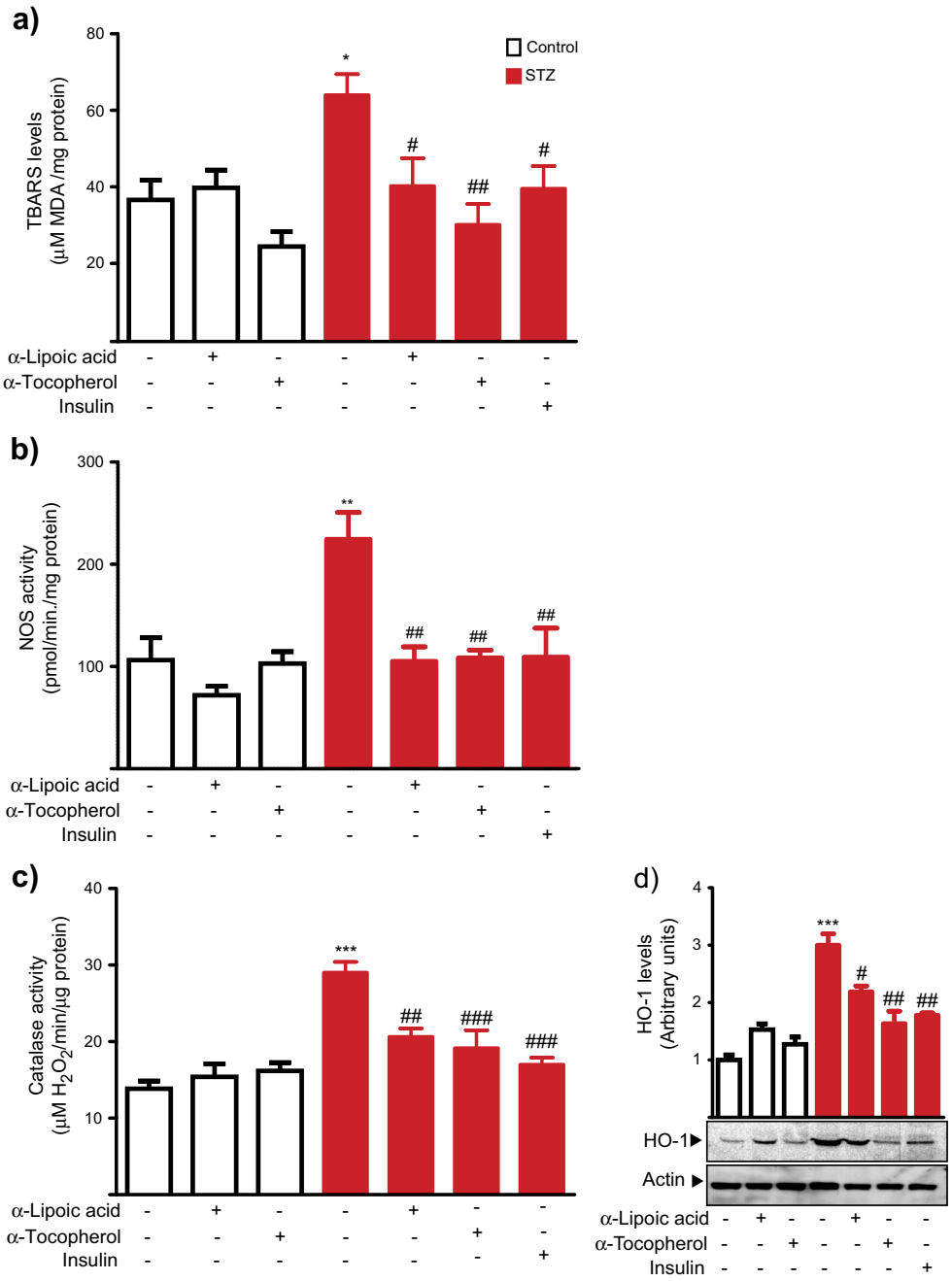

Fig. 3 Antioxidant treatment prevented changes in oxidative stress parameters and in the activity of antioxidant enzymes in adrenocortical tissue. Subgroups of control (white bars) and STZ-induced diabetic rats (red bars) received either α-lipoic acid (90 mg/kg ip, every 48 h) or α-tocopherol (200 mg/kg po, every 48 h) throughout the experimental period. Another group of STZ-treated rats were implanted with an insulin pellet (2.5 U insulin/day) after the confirmation of hyperglycemia. The following parameters were determined: **a** Lipid peroxidation levels evaluated as TBARS, **b** NOS activity, **c** catalase activity, and **d** Heme oxygenase expression levels. Data are shown as mean ± SEM, $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ vs. control, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, and $^{\#\#\#}p < 0.001$ vs. STZ (ANOVA followed by Tukey's test)

glucocorticoids are necessary for the initial metabolic and immune responses to stress, whereas higher levels may inhibit cellular and tissue reactions with harmful effects on the individual [32]. In fact, a suppressed adrenal function, as determined in pathological situations (stress or illness) may be lethal [33]. In this context, the discovery of new therapies that could help to overcome adrenocortical insufficiency in coping with stressful situations acquires relevance.

The results presented in this paper show that insulinopenic-diabetes, induced by STZ treatment in rats, is associated with higher basal serum corticosterone levels along with lower plasma ACTH concentrations. In agreement, using alloxan to induce beta-cell death, another model of type 1 diabetes [34, 35], higher basal corticosterone levels were also reported.

Based on the diminished response to exogenous ACTH administration, we hypothesize that insulin deprivation affects the steroidogenic capacity of the adrenal gland, suggesting that steroidogenic cells are also susceptible to the metabolic derangements triggered by the chronic lack of insulin, among them, a sustain exposure to significantly elevated glucose levels, the main cause of long term

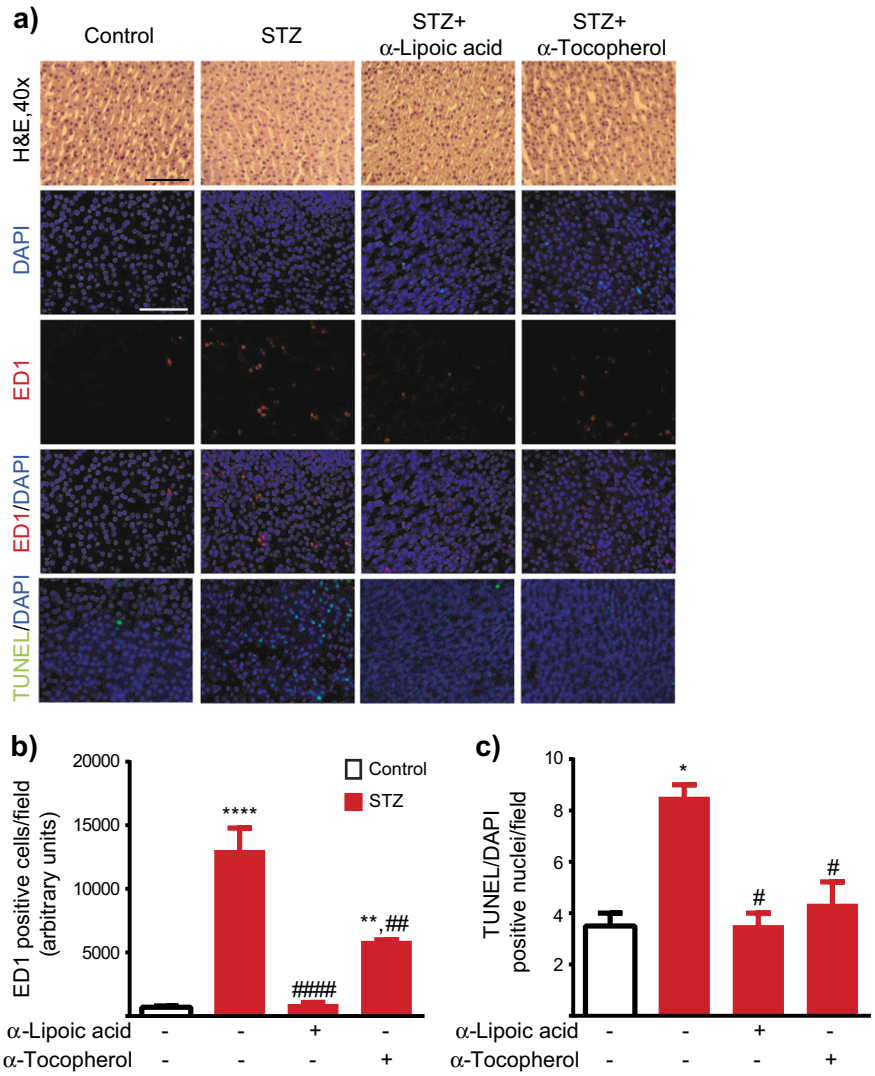

Fig. 4 Antioxidant treatment prevents the induction of apoptosis and phagocytosis in the adrenal cortex of STZ-induced diabetic rats. **a** Representative photomicrographs of H&E stained adrenal sections from Control, STZ and antioxidant + STZ-treated rats (40x magnification, upper panels, black bar = 20 µm). Photomicrographs of nuclei staining (DAPI, blue) and ED1 immunostaining (red) in flat-mounted adrenal sections (10×, scale white bar = 250 µm) are shown below. Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick-end labeling (TUNEL) staining of adrenal sections are shown in the lower panels (green). Quantification of ED1 positive signals per field (**b**) and TUNEL-positive signals relative to DAPI-stained nuclei per field (**c**) are presented as histograms. Data are shown as mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ vs. control, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, and $^{\#\#\#}p < 0.001$ vs. STZ (ANOVA followed by Tukey's test)

diabetic complications. Present results do not allow us to discard additional effects of STZ-induced diabetes at other levels of the HPA axis.

As for the decreased adrenocortical response in the ACTH stimulatory test, Chan et al. [36] obtained similar results in STZ-treated rats, after 14 days of diabetes induction. Moreover, in humans, several studies have shown a decrease in the adrenal response to ACTH in pediatric and young adults with type 1 diabetes [37–39]. Sharma et al. attributed these effects to glucotoxicity, binding of advanced glycation end products to ACTH receptors or altered glycosylation patterns of key proteins on zona fasciculata cells of the adrenal cortex in hyperglycemic subjects [39].

As many studies have demonstrated the association of hyperglycemia with the generation of oxidative stress [5] we sought to analyze indicative parameters in adrenocortical tissues. Although the adrenal cortex is equipped with several antioxidant molecules and enzyme systems [40, 41], we hereby demonstrated that induction of insulin-dependent diabetes is accompanied by an increase in the generation of lipoperoxides in addition to protein carbonyls [27], in spite of the higher activity of antioxidant enzymes as catalase and heme oxygenase detected four weeks after diabetes induction.

We also detected an increase in NOS activity in the adrenal cortex of STZ-induced diabetic rats, that was associated with higher expression levels of both NOS3 and NOS1 [27] and could be the result of the generation of oxidative stress, as was demonstrated by several authors [42, 43]. Peroxynitrite formed by the combination of NO with superoxide anions induced the increase in nitrotyrosine-modified proteins observed in these tissues. A decrease in NO availability could also upregulate NOS3 expression by relieving the inhibitory effect of NO, as suggested [42, 44, 45]. Superoxide anion could be

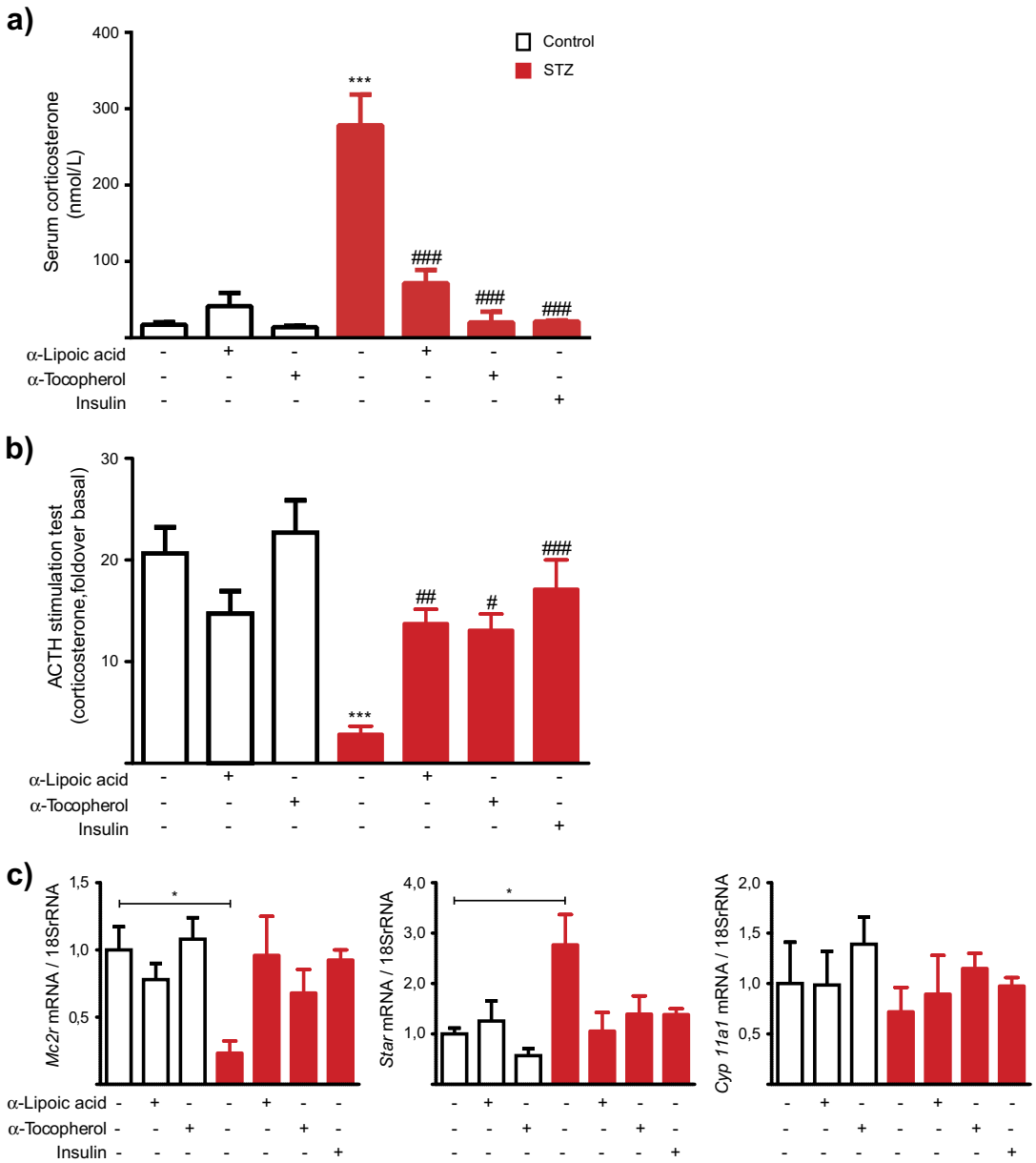

Fig. 5 Adrenocortical function was normalized by antioxidant treatment. Animals from Control (white bars) and STZ-groups (red bars) were treated as described in the legend of Fig. 3. Serum corticosterone levels were determined by radioimmunoassay in **a** basal conditions, and **b** before and 60 min after ACTH injection (7 IU/kg, ip). **c** mRNA levels of adrenal ACTH receptor (*Mc2r*), *Star* and *Cyp11a1* were determined by real-time PCR. Data are shown as mean ± SEM. $^{*}p < 0.05$, and $^{***}p < 0.001$ vs. control, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, and $^{\#\#\#}p < 0.001$ vs. STZ (ANOVA followed by Tukey's test)

generated by NADPH oxidase, the mitochondrial electron transport chain or uncoupled NOS activity as a result of the increased metabolic burden to the adrenal cortex [46].

As it is evident from the above described results, the endogenous antioxidant defense system, induced to some extent in STZ-induced diabetic rats, is overwhelm by the generation of reactive oxygen species (ROS) or reactive nitrogen species (RNS) and therefore its activity is not enough to prevent deleterious effects attributed to the consequences of insulin deprivation. Thus, the beneficial effect of antioxidant treatments in diabetic animals as demonstrated by several authors [8, 47–49]. Bearing this in mind, we speculated that these treatments could contribute to the amelioration of the adrenocortical dysfunction detected in STZ-induced diabetic rats. For these studies we chose two antioxidants with different biodistribution characteristics, α-lipoic acid and α-tocopherol, whose effectiveness in the treatment of diabetes have been previously determined [14, 50–52]. Although neither antioxidant was able to prevent the decrease in body weight, the increase in glycaemia or

the dyslipidemia detected after four weeks of STZ-treatment, as observed in other studies (α-tocopherol [53, 54] or α-lipoic acid [55, 56]), both molecules were able to improve the adrenocortical function.

Both antioxidants were effective in preventing the generation of oxidative stress in the adrenal cortex, as evidenced by their effects on lipoperoxide formation and in catalase, HO-1 and NOS activities. The effect on NOS activity (and hence in NO and NO-derived products) supports a causative link between oxidative stress and NOS induction. Similar results were obtained in kidneys from STZ- and Vitamin E- treated rats [50] and in the bronchial epithelium of diabetic animals, where an increase in NOS3 levels was not observed in α-lipoic acid treated rats [57].

Regarding adrenocortical function, both antioxidants prevented the decreased corticosterone response in the ACTH stimulation test, when the full capacity of the adrenal gland was evaluated. We thus hypothesize that adrenocortical insufficiency could be the consequence of the accumulation of deleterious effects of ROS and RNS on steroidogenic cells of the *zona fasciculata*, and may involve degeneration, phagocytosis, necrosis, and/or apoptosis [58–60]. As stated before, disruption of cellular homeostasis could generate oxidative stress leading to cell death by apoptosis [61, 62], and also increase phagocytic activity on dead cells and debris. In agreement, antioxidant treatment prevented the increase in the number of apoptotic and phagocytic cells. Similarly, α-lipoic acid has been shown to inhibit apoptosis in skeletal muscle of alloxan-induced diabetes in rats [63] or in HIT-T15 cells incubated in high-glucose media [64], while γ-tocopherol ameliorates this process in cutaneous wounds of diabetic mice [65]. As antioxidant treatments also preserved the steroidogenic response to an acute stimulation with ACTH we postulate an association of the induction of oxidative/ nitrosative stress leading to cell damage/death, with a loss of adrenal functional capacity.

A decrease in ACTH secretion could also be followed by atrophy and apoptosis of adrenocortical cells, an effect that has been associated with an increase in the number of local macrophages and phagocytic activity [66]. However, as antioxidant treatments had no effect on plasma ACTH levels in diabetic animals we suggest the prevalence of local effects on the observed adrenal dysfunction.

In spite of the lower plasma ACTH concentration detected in diabetic rats, basal corticosteronemia and *Star* mRNA levels were significantly higher than those of control rats. As this effect was also prevented by antioxidant treatment we suggest that regulatory signals associated with the generation of oxidative stress are involved in the modulation of steroid production. Among potential candidates, the stimulatory effect of different cytokines on adrenal steroidogenesis has been previously shown [67–70]. Increased systemic and tissue (retina, kidney, etc.) levels of several of these cytokines (IL1β, TNFα, IL6 among others) have been detected in diabetic rats [71, 72], while normalizing effects of antioxidants on their production have been also demonstrated [73, 74]. In addition to circulating cytokines, phagocytic macrophages (ED1 positive signals), as those detected in adrenocortical tissues from diabetic rats, have been also shown to produce cytokines [75]. Thus, the effect of inflammatory cytokines could significantly stimulate glucocorticoid production, and thus account for the observed discrepancy between high basal serum corticosterone levels along with low plasma ACTH concentrations. Identification of cytokines involved in the dysregulation of adrenal steroidogenesis in STZ-treated rats is currently under investigation.

In addition to the inhibitory effect on the production of pro-inflammatory cytokines, several studies have also demonstrated that glucocorticoids stimulate macrophage phagocytic activity on apoptotic cells [76, 77]. In agreement, in our study we showed an increase in the number of both apoptotic and active phagocytic cells in the adrenal cortex of diabetic rats. We postulate that ROS/RNS production leads to cell damage and apoptosis (and possibly other forms of cellular death) generating DAMPS (damage-associated molecular patterns) that are involved in macrophage activation within the tissue. In this context, corticosterone could contribute to an augmented macrophage phagocytic activity on apoptotic cells as we indicated before.

An increase in basal steroid levels could also be due to a stimulatory effect of LDL-cholesterol levels on *Star* expression, as was demonstrated in a mouse adrenocortical cell line [78]. Accordingly, we and others have shown an increase in this serum lipoprotein in STZ-treated rats after four weeks of treatment [79]. However, we ruled out this hypothesis, as antioxidant treatment had did not prevent the increase in non HDL-cholesterol levels in STZ-induced diabetic rats.

In summary, antioxidant treatment that prevented changes in ROS generation, NOS activity and the increase in the number of apoptotic and phagocytic cells, also restored the functional capacity of the adrenal gland. Our results provide, to our knowledge, the first evidences linking the generation of oxidative stress, apoptosis, and phagocytosis to the adrenocortical dysfunction determined in STZ-induced diabetic rats.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

References

1. J.L. Rains, S.K. Jain, Oxidative stress, insulin signaling, and diabetes. Free Radic. Biol. Med. **50**(5), 567–575 (2011)
2. D.A. Greene, M.J. Stevens, I. Obrosova, E.L. Feldman, Glucose-induced oxidative stress and programmed cell death in diabetic neuropathy. Eur. J. Pharmacol. **375**(1-3), 217–223 (1999)
3. P. Pacher, I.G. Obrosova, J.G. Mabley, C. Szabo, Role of nitrosative stress and peroxynitrite in the pathogenesis of diabetic complications. Emerging new therapeutical strategies. Curr. Med. Chem. **12**(3), 267–275 (2005)
4. T. Nishikawa, D. Edelstein, M. Brownlee, The missing link: a single unifying mechanism for diabetic complications. Kidney Int. Suppl. **77**, S26–30 (2000)
5. F. Giacco, M. Brownlee, Oxidative stress and diabetic complications. Circ. Res. **107**(9), 1058–1070 (2010)
6. E. Di Marco, J.C. Jha, A. Sharma, J.L. Wilkinson-Berka, K.A. Jandeleit-Dahm, J.B. de Haan, Are reactive oxygen species still the basis for diabetic complications? Clin. Sci. **129**(2), 199–216 (2015)
7. R. Brito, G. Castillo, J. Gonzalez, N. Valls, R. Rodrigo, Oxidative stress in hypertension: mechanisms and therapeutic opportunities. Exp. Clin. Endocrinol. Diabetes **123**(6), 325–335 (2015)
8. C. Karasu, Glycoxidative stress and cardiovascular complications in experimentally-induced diabetes: effects of antioxidant treatment. Open Cardiovasc. Med. J. **4**, 240–256 (2010)
9. L. Rochette, S. Ghibu, A. Muresan, C. Vergely, Alpha-lipoic acid: molecular mechanisms and therapeutic potential in diabetes. Can. J. Physiol. Pharmacol. **93**(12), 1021–1027 (2015)
10. H. Goldenstein, N.S. Levy, Y.T. Lipener, A.P. Levy, Patient selection and vitamin E treatment in diabetes mellitus. Expert. Rev. Cardiovasc. Ther. **11**(3), 319–326 (2013)
11. K. Petersen Shay, R.F. Moreau, E.J. Smith, T.M. Hagen, Is alpha-lipoic acid a scavenger of reactive oxygen species in vivo? Evidence for its initiation of stress signaling pathways that promote endogenous antioxidant capacity. IUBMB Life **60**(6), 362–367 (2008)
12. H. Moini, L. Packer, N.E. Saris, Antioxidant and prooxidant activities of alpha-lipoic acid and dihydrolipoic acid. Toxicol. Appl. Pharmacol. **182**(1), 84–90 (2002)
13. M. Whiteman, H. Tritschler, B. Halliwell, Protection against peroxynitrite-dependent tyrosine nitration and alpha 1-antiproteinase inactivation by oxidized and reduced lipoic acid. FEBS Lett. **379**(1), 74–76 (1996)
14. L. Packer, E.H. Witt, H.J. Tritschler, alpha-Lipoic acid as a biological antioxidant. Free Radic. Biol. Med. **19**(2), 227–250 (1995)
15. V.E. Kagan, A. Shvedova, E. Serbinova, S. Khan, C. Swanson, R. Powell, L. Packer, Dihydrolipoic acid—a universal antioxidant both in the membrane and in the aqueous phase. Reduction of peroxyl, ascorbyl and chromanoxyl radicals. Biochem. Pharmacol. **44**(8), 1637–1649 (1992)
16. S. Golbidi, M. Badran, I. Laher, Diabetes and alpha lipoic acid. Front. Pharmacol. **2**, 69 (2011)
17. T. Tankova, D. Koev, L. Dakovska, Alpha-lipoic acid in the treatment of autonomic diabetic neuropathy (controlled, randomized, open-label study). Rom. J. Intern. Med. **42**(2), 457–464 (2004)
18. T. Varkonyi, A. Korei, Z. Putz, T. Martos, K. Keresztes, C. Lengyel, S. Nyiraty, A. Stirban, G. Jermendy, P. Kempler, Advances in the management of diabetic neuropathy. Minerva Med. **108**(5), 419–437 (2017)
19. D. Ziegler, A. Ametov, A. Barinov, P.J. Dyck, I. Gurieva, P.A. Low, U. Munzel, N. Yakhno, I. Raz, M. Novosadova, J. Maus, R. Samigullin, Oral treatment with alpha-lipoic acid improves symptomatic diabetic polyneuropathy: the SYDNEY 2 trial. Diabetes Care **29**(11), 2365–2370 (2006)
20. J.W. Chang, E.K. Lee, T.H. Kim, W.K. Min, S. Chun, K.U. Lee, S.B. Kim, J.S. Park, Effects of alpha-lipoic acid on the plasma levels of asymmetric dimethylarginine in diabetic end-stage renal disease patients on hemodialysis: a pilot study. Am. J. Nephrol. **27**(1), 70–74 (2007)
21. M. Morcos, V. Borcea, B. Isermann, S. Gehrke, T. Ehret, M. Henkels, S. Schiekofer, M. Hofmann, J. Amiral, H. Tritschler, R. Ziegler, P. Wahl, P.P. Nawroth, Effect of alpha-lipoic acid on the progression of endothelial cell damage and albuminuria in patients with diabetes mellitus: an exploratory study. Diabetes Res. Clin. Pract. **52**(3), 175–183 (2001)
22. S.K. Hegazy, O.A. Tolba, T.M. Mostafa, M.A. Eid, D.R. El-Afify, Alpha-lipoic acid improves subclinical left ventricular dysfunction in asymptomatic patients with type 1 diabetes. Rev. Diabet. Stud. **10**(1), 58–67 (2013)
23. G.W. Burton, K.U. Ingold, Vitamin E as an in vitro and in vivo antioxidant. Ann. N. Y. Acad. Sci. **570**, 7–22 (1989)
24. P.G. Khatami, A. Soleimani, N. Sharifi, E. Aghadavod, Z. Asemi, The effects of high-dose vitamin E supplementation on biomarkers of kidney injury, inflammation, and oxidative stress in patients with diabetic nephropathy: a randomized, double-blind, placebo-controlled trial. J. Clin. Lipidol. **10**(4), 922–929 (2016)
25. S. Gupta, T.K. Sharma, G.G. Kaushik, V.P. Shekhawat, Vitamin E supplementation may ameliorate oxidative stress in type 1 diabetes mellitus patients. Clin. Lab. **57**(5-6), 379–386 (2011)
26. C. Giannini, F. Lombardo, F. Curro, M. Pomilio, T. Bucciarelli, F. Chiarelli, A. Mohn, Effects of high-dose vitamin E supplementation on oxidative stress and microalbuminuria in young adult patients with childhood onset type 1 diabetes mellitus. Diabetes Metab. Res. Rev. **23**(7), 539–546 (2007)
27. E.M. Repetto, R. Sanchez, J. Cipelli, F. Astort, C.M. Calejman, G. G. Piroli, P. Arias, C.B. Cymeryng, Dysregulation of corticosterone secretion in streptozotocin-diabetic rats: modulatory role of the adrenocortical nitrergic system. Endocrinology **151**(1), 203–210 (2010)
28. D.D. Wink, S. Kim, E. Coffin, J. Cook, Y. Vodovotz, D. Chistodoulou, D. Jourd'heuil, M. Grisham, Detection of S-nitrosothiols by fluorometric and colorimetric methods. Methods Enzymol. **301**, 201–211 (1999). https://doi.org/10.1016/S0076-6879(99)01083-6
29. Y. Pomeraniec, N. Grion, L. Gadda, V. Pannunzio, E.J. Podesta, C.B. Cymeryng, Adrenocorticotropin induces heme oxygenase-1 expression in adrenal cells. J. Endocrinol. **180**(1), 113–124 (2004)
30. H. Aebi. Catalase. *Methods of Enzymatic Analysis*. H. Bermeyer (ed.) Verlag Chemie, Wenheim), 1982) 271–282
31. M.E. Mercau, F. Astort, E.F. Giordanino, C. Martinez Calejman, R. Sanchez, L. Caldareri, E.M. Repetto, O.A. Coso, C.B. Cymeryng, Involvement of PI3K/Akt and p38 MAPK in the induction of COX-2 expression by bacterial lipopolysaccharide in murine adrenocortical cells. Mol. Cell. Endocrinol. **384**(1-2), 43–51 (2013)
32. S.A. Motzer, V. Hertig, Stress, stress response, and health. Nurs. Clin. N. Am. **39**(1), 1–17 (2004)
33. T.H. Puar, N.M. Stikkelbroeck, L.C. Smans, P.M. Zelissen, A.R. Hermus, Adrenal crisis: still a deadly event in the 21st century. Am. J. Med. **129**(3), 339 e331–339 (2016)
34. P.K. Singh, D. Baxi, S. Banerjee, A.V. Ramachandran, Therapy with methanolic extract of Pterocarpus marsupium Roxb and Ocimum sanctum Linn reverses dyslipidemia and oxidative stress

in alloxan induced type I diabetic rat model. Exp. Toxicol. Pathol. **64**(5), 441–448 (2010)
35. I.G. Danilova, T.S. Bulavintceva, I.F. Gette, S.Y. Medvedeva, V. V. Emelyanov, M.T. Abidov, Partial recovery from alloxan-induced diabetes by sodium phthalhydrazide in rats. Biomed. Pharmacother. **95**, 103–110 (2017)
36. O. Chan, K. Inouye, M. Vranic, S.G. Matthews, Hyperactivation of the hypothalamo-pituitary-adrenocortical axis in streptozotocin-diabetes is associated with reduced stress responsiveness and decreased pituitary and adrenal sensitivity. Endocrinology **143**(5), 1761–1768 (2002)
37. K. Simunkova, R. Hampl, M. Hill, L. Kriz, P. Hrda, D. Janickova-Zdarska, V. Zamrazil, J. Vrbikova, K. Vondra, Adrenocortical function in young adults with diabetes mellitus type 1. J. Steroid Biochem. Mol. Biol. **122**(1-3), 35–41 (2010)
38. X. Gaete, G. Iniguez, J. Linares, A. Avila, V. Mericq, Cortisol hyporesponsiveness to the low dose ACTH test is a frequent finding in a pediatric population with type 1 diabetes mellitus. Pediatr. Diabetes **14**(6), 429–434 (2010)
39. A.N. Sharma, J. Wigham, J.D. Veldhuis, Corticotropic axis drive of overnight cortisol secretion is suppressed in adolescents and young adults with type 1 diabetes mellitus. Pediatr. Diabetes **15** (6), 444–452 (2014)
40. S. Azhar, L. Cao, E. Reaven, Alteration of the adrenal antioxidant defense system during aging in rats. J. Clin. Invest. **96**(3), 1414–1424 (1995)
41. P. Patak, H.S. Willenberg, S.R. Bornstein, Vitamin C is an important cofactor for both adrenal cortex and adrenal medulla. Endocr. Res. **30**(4), 871–875 (2004)
42. J. Zhen, H. Lu, X.Q. Wang, N.D. Vaziri, X.J. Zhou, Upregulation of endothelial and inducible nitric oxide synthase expression by reactive oxygen species. Am. J. Hypertens. **21**(1), 28–34 (2008)
43. J.H. Jang, J.N. Chun, S. Godo, G. Wu, H. Shimokawa, C.Z. Jin, J. H. Jeon, S.J. Kim, Z.H. Jin, Y.H. Zhang, ROS and endothelial nitric oxide synthase (eNOS)-dependent trafficking of angiotensin II type 2 receptor begets neuronal NOS in cardiac myocytes. Basic Res. Cardiol. **110**(3), 21 (2015)
44. N.D. Vaziri, Y. Ding, Z. Ni, Compensatory up-regulation of nitric-oxide synthase isoforms in lead-induced hypertension; reversal by a superoxide dismutase-mimetic drug. J. Pharmacol. Exp. Ther. **298**(2), 679–685 (2001)
45. G.M. Buga, J.M. Griscavage, N.E. Rogers, L.J. Ignarro, Negative feedback regulation of endothelial cell function by nitric oxide. Circ. Res. **73**(5), 808–812 (1993)
46. Y. Du, C.M. Miller, T.S. Kern, Hyperglycemia increases mitochondrial superoxide in retina and retinal cells. Free Radic. Biol. Med. **35**(11), 1491–1499 (2003)
47. R.A. Kowluru, R.L. Engerman, T.S. Kern, Diabetes-induced metabolic abnormalities in myocardium: effect of antioxidant therapy. Free Radic. Res. **32**(1), 67–74 (2000)
48. D. Koya, K. Hayashi, M. Kitada, A. Kashiwagi, R. Kikkawa, M. Haneda, Effects of antioxidants in diabetes-induced oxidative stress in the glomeruli of diabetic rats. J. Am. Soc. Nephrol. **14** (8Suppl 3), S250–253 (2003)
49. V.M. Altan, The pharmacology of diabetic complications. Curr. Med. Chem. **10**(15), 1317–1327 (2003)
50. M.A. Haidara, D.P. Mikhailidis, M.A. Rateb, Z.A. Ahmed, H.Z. Yassin, I.M. Ibrahim, L.A. Rashed, Evaluation of the effect of oxidative stress and vitamin E supplementation on renal function in rats with streptozotocin-induced Type 1 diabetes. J. Diabetes Complicat. **23**(2), 130–136 (2009)
51. S. Alireza, N. Leila, S. Siamak, K.A. Mohammad-Hasan, I. Behrouz, Effects of vitamin E on pathological changes induced by diabetes in rat lungs. Respir. Physiol. Neurobiol. **185**(3), 593–599 (2013)
52. M.B. Gomes, C.A. Negrato, Alpha-lipoic acid as a pleiotropic compound with potential therapeutic use in diabetes and other chronic diseases. Diabetol. Metab. Syndr. **6**(1), 80 (2014)
53. G. Nascimento Gomes, F.T. Barbosa, R.F. Radaeli, M.F. Cavanal, M. Mello Aires, F. Zaladek Gil, Effect of D-alpha-tocopherol on tubular nephron acidification by rats with induced diabetes mellitus. Braz. J. Med. Biol. Res. **38**(7), 1043–1051 (2005)
54. D. Comin, L. Gazarini, J.N. Zanoni, H. Milani, R.M. de Oliveira, Vitamin E improves learning performance and changes the expression of nitric oxide-producing neurons in the brains of diabetic rats. Behav. Brain. Res. **210**(1), 38–45 (2010)
55. I. Obrosova, X. Cao, D.A. Greene, M.J. Stevens, Diabetes-induced changes in lens antioxidant status, glucose utilization and energy metabolism: effect of DL-alpha-lipoic acid. Diabetologia **41**(12), 1442–1450 (1998)
56. A.C. Maritim, R.A. Sanders, J.B. Watkins, 3rd: effects of alpha-lipoic acid on biomarkers of oxidative stress in streptozotocin-induced diabetic rats. J. Nutr. Biochem. **14**(5), 288–294 (2003)
57. C. Hurdag, I. Uyaner, E. Gurel, A. Utkusavas, P. Atukeren, C. Demirci, The effect of alpha-lipoic acid on NOS dispersion in the lung of streptozotocin-induced diabetic rats. J. Diabetes Complicat. **22**(1), 56–61 (2008)
58. S. Xu, C. Chen, W.X. Wang, S.R. Huang, J. Yu, X.Y. Chen, IIA phospholipase A2 in pancreatitis-associated adrenal injury in acute necrotizing pancreatitis. Pathol. Res. Pract. **206**(2), 73–82 (2010).
59. J. Yu, S. Xu, W.X. Wang, W.H. Deng, H. Jin, X.Y. Chen, C. Chen, H.T. Sun, Changes of inflammation and apoptosis in adrenal gland after experimental injury in rats with acute necrotizing pancreatitis. Inflammation **35**(1), 11–22 (2012)
60. J. Yu, T. Zuo, W. Deng, Q. Shi, P. Ma, C. Chen, L. Zhao, K. Zhao, W. Wang, Poly(ADP-ribose) polymerase inhibition suppresses inflammation and promotes recovery from adrenal injury in a rat model of acute necrotizing pancreatitis. Bmc. Gastroenterol. **16**(1), 81 (2016)
61. A. Kupsco, D. Schlenk, Oxidative stress, unfolded protein response, and apoptosis in developmental toxicity. Int. Rev. Cell. Mol. Biol. **317**, 1–66 (2011)
62. C.J. Norbury, I.D. Hickson, Cellular responses to DNA damage. Annu. Rev. Pharmacol. Toxicol. **41**, 367–401 (2001)
63. R. Saraswathi, S.N. Devaraj, Oxidative stress in skeletal muscle impairs mitochondrial function in alloxan induced diabetic rats: role of alpha lipoic acid. Biomed. Prev. Nutr. **3**(3), 213–219 (2013). https://doi.org/10.1016/j.bionut.2012.08.006
64. Y. Yang, W. Wang, Y. Liu, T. Guo, P. Chen, K. Ma, C. Zhou, Alpha-lipoic acid inhibits high glucose-induced apoptosis in HIT-T15 cells. Dev. Growth Differ. **54**(5), 557–565 (2012)
65. J. Shin, S.J. Yang, Y. Lim, Gamma-tocopherol supplementation ameliorated hyper-inflammatory response during the early cutaneous wound healing in alloxan-induced diabetic mice. Exp. Biol. Med. **242**(5), 505–515 (2017)
66. H. Almeida, J. Ferreira, D. Neves, Macrophages of the adrenal cortex: a morphological study of the effects of aging and dexamethasone administration. Ann. N. Y. Acad. Sci. **1019**, 135–140 (2004)
67. S. McIlmoil, J. Strickland, A.M. Judd, Interleukin 6 increases the in vitro expression of key proteins associated with steroidogenesis in the bovine adrenal zona fasciculata. Domest. Anim. Endocrinol. **55**, 11–24 (2016)
68. A.V. Turnbull, C.L. Rivier, Regulation of the hypothalamic-pituitary-adrenal axis by cytokines: actions and mechanisms of action. Physiol. Rev. **79**(1), 1–71 (1999)
69. S.R. Bornstein, H. Rutkowski, I. Vrezas, Cytokines and steroidogenesis. Mol. Cell. Endocrinol. **215**(1-2), 135–141 (2004)
70. I.V. Tkachenko, T. Jaaskelainen, J. Jaaskelainen, J.J. Palvimo, R. Voutilainen, Interleukins 1alpha and 1beta as regulators of

steroidogenesis in human NCI-H295R adrenocortical cells. Steroids **76**(10-11), 1103–1115 (2011)
71. A. Hasanvand, H. Amini-Khoei, M.R. Hadian, A. Abdollahi, S.M. Tavangar, A.R. Dehpour, E. Semiei, S.E. Mehr, Anti-inflammatory effect of AMPK signaling pathway in rat model of diabetic neuropathy. Inflammopharmacology **24**(5), 207–219 (2016)
72. J.F. Navarro-Gonzalez, C. Mora-Fernandez, The role of inflammatory cytokines in diabetic nephropathy. J. Am. Soc. Nephrol. **19**(3), 433–442 (2008)
73. R.A. Kowluru, Q. Zhong, J.M. Santos, M. Thandampallayam, D. Putt, D.L. Gierhart, Beneficial effects of the nutritional supplements on the development of diabetic retinopathy. Nutr. Metab. **11** (1), 8 (2014)
74. T. Ono, S. Takada, S. Kinugawa, H. Tsutsui, Curcumin ameliorates skeletal muscle atrophy in type 1 diabetic mice by inhibiting protein ubiquitination. Exp. Physiol. **100**(9), 1052–1063 (2015)
75. M.B. Ka, A. Daumas, J. Textoris, J.L. Mege, Phenotypic diversity and emerging new tools to study macrophage activation in bacterial infectious diseases. Front. Immunol. **5**, 500 (2014)
76. A. Nilsson, L. Vesterlund, P.A. Oldenborg, Macrophage expression of LRP1, a receptor for apoptotic cells and unopsonized erythrocytes, can be regulated by glucocorticoids. Biochem. Biophys. Res. Commun. **417**(4), 1304–1309 (2012)
77. J.S. Gilmour, A.E. Coutinho, J.F. Cailhier, T.Y. Man, M. Clay, G. Thomas, H.J. Harris, J.J. Mullins, J.R. Seckl, J.S. Savill, K.E. Chapman, Local amplification of glucocorticoids by 11 beta-hydroxysteroid dehydrogenase type 1 promotes macrophage phagocytosis of apoptotic leukocytes. J. Immunol. **176**(12), 7605–7611 (2006)
78. M.E. Reyland, R.M. Evans, E.K. White, Lipoproteins regulate expression of the steroidogenic acute regulatory protein (StAR) in mouse adrenocortical cells. J. Biol. Chem. **275**(47), 36637–36644 (2000)
79. G.S. Prasath, S.P. Subramanian, Antihyperlipidemic effect of fisetin, a bioflavonoid of strawberries, studied in streptozotocin-induced diabetic rats. J. Biochem. Mol. Toxicol. **28**(10), 442–449 (2014)