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ORIGINAL CONTRIBUTION

Alpha-lipoic acid preserves the structural and functional integrity of red blood cells by adjusting the redox disturbance and decreasing O-GlcNAc modifications of antioxidant enzymes and heat shock proteins in diabetic rats

Mihailović Mirjana · Arambašić Jelena · Uskoković Aleksandra · Dinić Svetlana · Grdović Nevena · Marković Jelena · Poznanović Goran · Vidaković Melita

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Abstract
Purpose The aim of this study was to investigate whether the daily administration of α-lipoic acid (LA) during 4 weeks prevents the redox disturbance in red blood cells (RBC) described in diabetes
Methods Multiple low-dose streptozotocin (STZ) diabetes was induced in rats by the administration of 40 mg/kg STZ intraperitoneally (i.p.) for 5 consecutive days. LA was applied at a dose of 10 mg/kg i.p. for 4 weeks, starting from the last day of STZ administration.
Results The LA-treated diabetic rats exhibited a general systemic improvement, revealed as the near restoration of body weight and of essential biochemical parameters. The latter was displayed as decreased hyperglycemia, lower triglyceride levels and lower serum activities of alanine aminotransferases and aspartate aminotransferases that point to a general improvement of diabetes-linked organ "lesions". The LA-treated diabetic rats also exhibited significant alleviation of oxidative stress, manifested as decreased lipid peroxidation and lower glycation levels of serum proteins and hemoglobin, while the RBC exhibited increased activities of antioxidant enzymes and elevated levels of reduced glutathione. In RBC, this was accompanied by decreased post-translational glycosylation by O-bound *β-N*-acetylglucosamine (O-GlcNAc) of the antioxidant enzymes superoxide dismutase and catalase and of heat shock proteins HSP70 and HSP90.
Conclusion LA through its powerful antioxidant activity preserves the structural and functional integrity of RBC in diabetes. The RBC can then assume a more efficient role as the first line of systemic defense against diabetic complications arising from oxidative stress–induced damage of other tissues and organs.

Keywords Red blood cells · Diabetes · Lipoic acid · O-GlcNAc · Antioxidative enzymes · Heat shock proteins

M. Mirjana (✉) · A. Jelena · U. Aleksandra · D. Svetlana · G. Nevena · M. Jelena · P. Goran · V. Melita
Institute for Biological Research Siniša Stanković, University of Belgrade, Bulevar despota Stefana 142, 11060 Belgrade, Serbia
e-mail: mista@ibiss.bg.ac.rs

Introduction

Diabetes mellitus (DM) is a metabolic disorder characterized by hyperglycemia, target-tissue resistance to insulin and insufficient insulin secretion [1]. High glucose concentrations promote increased formation of toxic reactive oxygen species (ROS) via glucose autoxidation [1], non-enzymatic protein glycation [2], increased metabolism of glucose through the hexosamine pathway [3], excessive activation of the polyol pathway by unused glucose and through glucose-mediated activation of protein kinase C [4]. Other important conditions related to diabetes are dyslipidemia and a number of pathological complications due to perturbations in the antioxidant defense network [5, 6]. The microvascular complications usually observed in diabetes, such as nephropathy, retinopathy and neuropathy, exacerbate these disturbances.

In the hyperglycemic environment, red blood cells (RBC) are subjected to compositional changes and are affected at the functional level [7]. RBC contribute to about 40% of the blood volume and are the first cellular structures to respond to increased ROS activity. Thus, the overall degree of RBC damage is a reflection of the general state of oxidative stress in the whole organism [8]. The

iron-containing oxygen carrier protein hemoglobin (Hb) is central to RBC functioning [9]. Under diabetic conditions, RBC dysfunction is linked to increased levels of methemoglobin (metHb) where the iron in the heme group is in the ferric state (Fe^{3+}), not in the ferrous (Fe^{2+}) state of normal Hb. The hyperglycemic conditions in diabetes also cause increased non-enzymatic glycation of Hb, resulting in significantly elevated levels of glycated Hb (GlyHb). The glycation of Hb increases its susceptibility to proteolytic degradation [10], alters the functioning of Hb by increasing its oxygen affinity and increases erythrocyte adherence to endothelial cells and erythrocyte turnover [11].

A feature of fully mature mammalian RBC is the absence of a nucleus. As a result, RBC cannot respond to increased ROS levels by upregulating the expression of antioxidant defense proteins. Instead, they rely on pre-existing proteins for protection against ROS damage [12]. RBC lack mitochondria, and the major source of ROS in RBC is the oxygenated form of Hb (HbO_2), which is easily oxidized to its ferric metHb form with the generation of superoxide anion (O_2^-). RBC continuously transport oxygen over the course of their 120-day lifespan and are exposed to some of the highest levels of oxidative stress in the body. Consequently, the RBC developed a system of defenses that represent an excellent example of redox balance maintenance [9]. They possess an extensive array of antioxidants, including membrane oxidoreductases, cellular antioxidants such as catalase (CAT) and superoxide dismutase (SOD) and the glutathione (GSH) system [13]. In addition to this system, the antioxidant functions of heat shock proteins (HSP) have also been assumed to be helpful in fighting diabetic complications, as numerous defects in the function of HSP appear to contribute to the commonly observed complications in diabetics [14]. They prevent protein aggregation, facilitate the folding of other cellular proteins and target improperly folded proteins to specific pathways for degradation [17]; therefore, the HSP assume an important role in the maintenance of homeostatic tissue protein functioning.

In addition to diabetes-related studies of endogenous mechanisms of ROS quenching, much attention has been focused on the pharmacology of antioxidant compounds in the treatment for diabetes. α-Lipoic acid (LA) is an example of a compound that possesses a therapeutic effect, which is related to its antioxidant activity [16]. LA is a nutritional dithiol and an essential, naturally occurring cofactor of mitochondrial dehydrogenase complexes [17]. It acts by scavenging free radicals, chelating metal ions and recycling antioxidants [18]. The therapeutic use of LA in preventing and treating diabetes-induced oxidative stress has been described [19, 20]. In clinical trials, LA improved glucose metabolism in patients with type 2 diabetes. LA has been in clinical use in Germany for more than 30 years for treating patients with diabetic neuropathy. Exogenously applied LA exhibits beneficial effects on dysfunctioning endothelial cells, neurons and muscles by preventing and reversing the development of diabetic complications [21].

In the present study, we examined the effects of LA on the redox status of RBC during streptozotocin (STZ)-induced diabetes in the rat. We present evidence that LA reverses the oxidative status in diabetes-induced dysfunction of RBC. Substantial reduction of hyperglycemic conditions and alleviation of oxidative stress in LA-treated diabetic rats were accompanied by a decreased level of lipid peroxidation, restitution of the GSH system and reduction of O-GlcNAcylation of antioxidant enzymes and HSP. The LA-induced reversal of the oxidative status in diabetic rats was observed as a normalization of antioxidant enzyme activities and increased HSP protein levels in RBC. These results show that in diabetes, LA plays an important role in reestablishing normal functioning of RBC, which assume a central function in systemic antioxidant protection of other tissues and organs.

Materials and methods

Animals

Experiments were performed on 2.5-month-old adult albino Wistar rats weighing 220–250 g. All the animal procedures were approved by the Committee for Ethical Animal Care and Use of the Institute for Biological Research, Belgrade, which acts in accordance with the Guide for the Care and Use of Laboratory Animals, published by the US National Institute of Health (NIH Publication No. 85/23, revised in 1986).

Experimental protocol

The experimental model of multiple low-dose STZ-induced diabetes was induced by intraperitoneal (i.p.) injection of Wistar rats with STZ at 40 mg/kg/day for 5 consecutive days. STZ was dissolved immediately before use in sodium citrate buffer (0.1 M, pH 4.5). Blood glucose was measured 24 h after the last STZ injection. Blood samples were obtained from the tail vein of overnight-fasted rats, and glucose was measured using a blood glucose meter (Accu-Chech Active) [22]. Rats were considered to have diabetes when the fasting blood glucose level exceeded 20 mmol/L. Male albino Wistar rats were randomly divided into four groups. (1) NDM—the non-diabetic group, also referred to as the negative control ($n = 7$), received an equivalent volume of citrate buffer i.p. for 5 consecutive days. (2) NDM + LA—the non-diabetic group, also referred to as the positive control ($n = 7$), was i.p. administered LA. LA was obtained from the company

"Ivančić i sinovi", Belgrade, Serbia. LA was administered daily at a dose of 10 mg/kg, as described by Maritim et al. [16], throughout the 4-week period. (3) DM—the diabetic group ($n = 8$) that was injected STZ (40 mg/kg/5 days, i.p.) and left untreated throughout the 4-week period. (4) DM + LA—the diabetic group (as described previously) that was treated with LA ($n = 8$); these rats received a daily i.p. dose of 10 mg/kg LA for 4 weeks, starting from the last day of STZ administration.

The body weight of all four experimental groups was recorded at the beginning of the experiments and 4 weeks after the treatment. The LA treatment of diabetic rats lowered the decline in animal body weight that was observed in the untreated diabetic group (data not shown).

Serum and hemolysate preparation

Rat blood serum was collected after blood clotting and centrifugation at 2,000×*g* for 10 min. The serum was used for the determination of α_2-macroglobulin (α_2M) and albumin concentrations, the iron content, the level of glycated proteins and the amount of protein sulfhydryl groups.

For the preparation of RBC hemolysates, blood was collected in heparinized tubes (1,000 IU of heparin) and centrifuged at 2,000×*g* for 10 min. RBC were washed twice with 0.9% NaCl and centrifuged under the same condition. The washed erythrocytes were lysed with 3 volume of cold water for 30 min on ice. Hemolysates were used for the determination of CAT, SOD and glutathione-S-transferase (GST) activities, the determination of thiobarbituric acid–reactive substance (TBARS), GSH and glutathionylated proteins (GSSP), Hb, GlyHb and metHb, as well as the level of O-GlcNAc-modified proteins.

Determination of the biochemical parameters of diabetes

Blood glucose levels were measured using a commercial kit (Gluco-quant Glucose/HK, Boehringer Mannheim, Germany) based on the hexokinase/G6P-DH enzymatic method. Hb was determined according to Drabkin and Austin [23]. GlyHb was determined by the colorimetric assay according to Parker et al. [24]. MetHb was determined according to the modification of the method of Dubowski [25], as described in Kawatsu et al. [26]. Activities of the alanine aminotransferases (ALT) and aspartate aminotransferases (AST) were estimated in the serum by measuring the produced oxaloacetate and pyruvate, respectively, using an optimized standard UV kinetic method kit (GPT (ALAT) IFCC mod., GOT (ASAT) IFCC mod). Serum triglycerides were measured by the GPO-PAP method with an enzymatic kit (Randox Laboratories, UK).

Reducing potential of LA

The reducing potential of LA was determined by the method of Oyaizu [27]. Different concentrations of LA (0.25–5 mg/mL) in 0.5 mL of 50% ethanol were mixed with phosphate buffer (0.5 mL, 0.2 M, pH 6.6) and potassium hexacyanoferrate [$K_3Fe(CN)_6$] (0.5 mL, 0.1%). After incubation, 0.5 mL of trichloroacetic acid (10%) was added to stop the reaction, and the samples were centrifuged for 10 min at 1,000×*g*. The upper layer of the solution (1 mL) was mixed with distilled water (1 mL) and $FeCl_3$ (0.1 mL, 0.01%). The absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicates greater reducing power. Ascorbic acid was used as a positive control.

Fe^{2+} chelation

The chelating activity of LA for ferrous ions was estimated by the method of Dinis et al. [28]. Briefly, different concentrations of LA (0.5–5 mg/mL) were dissolved in 0.25 mL of 0.125 mM $FeSO_4$. The reaction was initiated by the addition of 0.3125 mM ferrozine (0.25 mL). The mixture was shaken vigorously and left standing at room temperature for 10 min. Absorbance of the solution was measured spectrophotometrically at 562 nm. The percentage of inhibition of the ferrozine–Fe^{2+} complex formation was calculated using the formula: Ferrous ions chelating effect (%) = $[(A_{\text{control}} - A_{\text{sample}})/A_{\text{control}}] \times 100$. EDTA was used as positive control.

Determination of serum DPPH activity

The ability of the serum to inhibit the DPPH (1,1-diphenyl-2-picrylhydrazyl) radical was measured according to Yokozawa et al. [29]. Rat serum (20 μL) was added to 3 mL of DPPH solution (0.05 mmol/L), and the reaction mixture was shaken vigorously. After incubation at room temperature for 20 min, the absorbance of the solution was determined at 517 nm. The DPPH solution without serum served as the control, and the ascorbic acid was used as a standard; all tests were performed in triplicate. The radical-scavenging activity of the tested samples, expressed as percentage inhibition of DPPH, was calculated according to the formula: (%) = $[(A_{\text{blanc}} - A_{\text{sample}})/A_{\text{blamc}}] \times 100$; A_{blanc} is the absorbance of the DPPH in solution without the test sample and A_{sample} is the absorbance of DPPH in the solution with the test sample.

Detection of glycated serum proteins by the fructosamine assay

The glycation of serum proteins was measured according to Johnson et al. [30]. Aliquots of sera (50 μL) were added to

450 μL of 100 mM carbonate buffer (pH 10.8) containing 0.5 mM nitro blue tetrazolium (NBT). The samples were incubated for 1 h at 37 °C. Absorbance was measured at 595 nm.

Determination of the iron content in the serum

The serum iron content was evaluated using the Ferrimat-Kit (bioMerieux SA, Marcy-l'Etoile, France). The ferrous iron forms a colored complex with ferrozine. The intensity of the color at 590 nm is directly proportional to the iron concentration in the sample. In order to determine the concentration of free ferrous iron (vs. total iron) in the serum, we conducted the same procedure as for total iron determination, but excluded the reductant hydroxylamine from the assay procedure.

Determination of α_2M and albumin levels in the serum

The concentration of serum α_2M was determined by rocket immunoelectrophoresis with anti-human α_2M antibody (Sigma-Aldrich) [31]. Serum albumin levels were determined according to Gambal [32]. The serum was treated with polyethylene glycol (25%, v/v) at 4 °C for 24 h in order to precipitate all other serum proteins except albumin that remained in the supernatant, followed by 20-min centrifugation at 30,000×*g* (Beckman, rotor Ti-50). The concentration of albumin was determined in the obtained supernatant, according to Lowry [33].

TBARS assay

The TBARS method was performed according to Ohkawa et al. [34]. Briefly, an aliquot of the hemolysates (0.1 mL) was mixed with 0.2 mL of 8.1% SDS, 1.5 mL of 20% acetic acid (pH 3.5), 1.5 mL of 0.8% TBA and 0.7 mL of water and heated at 95 °C for 60 min. After cooling to room temperature, 1 mL of water and 5 mL of n-butanol/pyridine (15:1, v/v) were added, mixed and centrifuged at 3,000×*g* for 10 min. The red pigment in the supernatant fractions was estimated by absorbance at 532 nm. A calibration curve was prepared with malondialdehyde (MDA) standard (MDA concentrations ranged from 25 nmol/mL to 1 μmol/mL).

Determination of protein sulfhydryl (SH) groups in the serum

Protein SH groups were determined by Ellman's method [35]. Briefly, 0.5 mL of serum was added to a cuvette containing 0.1 M phosphate buffer, pH 7.4 (0.5 mL); 0.2 mL of 3 mM 5,5-dithiobis (2-nitrobenzoic acid) was then added to start the reaction. After 10 min, absorbance was measured at 412 nm. The amount of SH groups was calculated according to the formula:

$$\text{molSH/gHb} = [(A_{\text{sample}}/14150) \times \text{dilutionfactor}]/\text{gHb}.$$

Determination of GSH and GSSP

Total GSH and oxidized GSH (GSSG) levels were determined colorimetrically using the Glutathion Assay Kit (Cayman Chemicals Company, Michigan, USA). The GSSG was then subtracted from the total GSH to determine the level of reduced GSH.

To determine GSSP, acid-precipitated proteins were thoroughly washed with the precipitating solution until no trace of soluble GSH or GSSG was detected. The pellets were then resuspended and brought to an alkaline pH (pH 7.5–8) for 5–30 min. Under these conditions, GSH is released via an–SH/–SS-exchange reaction. The reaction was stopped by the addition of trichloroacetic acid to a final 5% concentration. The amount of released GSH was determined enzymatically in the supernatants after centrifugation.

Determination of SOD, CAT, and GST activities

Hemolysates of RBC were used for the determination of GST activity [36]. One unit of GST activity was defined as nmol GSH/min/mL of plasma. CAT activity was determined according to Beutler [37] by the rate of hydrogen peroxide decomposition and expressed as μmol H_2O_2/min/g Hb. For the measurement of CuZnSOD activity, hemoglobin was previously removed from hemolysates and CuZnSOD activity was measured by the epinephrine method [38]. One unit of SOD activity was defined as the amount of Hb that causes 50% inhibition of adrenaline autoxidation.

Detection of O-GlcNAc glycosylation

Wheat germ lectin affinity purification of glycosylated species containing N-acetylglucosamine residues was performed according to Jackson and Tjian [39]. Equal amounts of hemolysates (1.5 mg) from all four experimental groups were submitted to chromatography on a column of wheat germ lectin immobilized on Sepharose (Pharmacia) at 4 °C. The column was prewashed with 50 mL of Z buffer (25 mM Hepes pH 7.6, 12.5 mM $MgCl_2$, 10% glycerol, 0.1% IGEPAL CA-630, 10 μM $ZnSO_4$ and 1 mM DTT) containing 0.1 M KCl. GlcNAc-containing glycoproteins were eluted with Z buffer containing 0.1 M KCl and N-acetylglucosamine (0.4 and 0.5 M). Equal aliquots of eluted proteins and hemolysates of all four

experimental groups were separated by 12% SDS–polyacrylamide gel electrophoresis (SDS-PAGE). The protein gels were subjected to silver staining or were electroblotted to PVDF membranes (Hybond-P, Amersham Pharmacia Biotech, Uppsala, Sweden) and analyzed by immunoblot analysis.

SDS–polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot analysis

Twenty micrograms of hemolysates were separated by SDS-PAGE [40]. Immunoblot analysis was performed by the procedure of Towbin et al. [41] using polyclonal antibodies to rat CuZnSOD, Hsp70, Hsp90 (Santa Cruz Biotechnology) and catalase (Abcam). Immunoreactive bands were identified by an enhanced chemiluminescence (ECL) detection system (Santa Cruz Biotechnology) according to the manufacturer's instructions.

Statistical analysis

Mean and standard error values (S.E.M.) were determined for all parameters studied. The results were statistically analyzed by analysis of variance (ANOVA). Duncan's multiple range test (DMRT) was performed to determine the significant difference between the groups.

Results

The biochemical parameters of diabetes

The major biochemical parameters of diabetes in all experimental groups are presented in Table 1. The blood glucose concentration significantly increased in the diabetic group (43.8 mmol/L). In the diabetic group treated with LA, the glucose concentration decreased to 19.9 mmol/L. The levels of AST, ALT and triglycerides were increased in the serum of rats from the untreated diabetic group twofold, threefold and sixfold, respectively, compared with the control group. As a result of the LA treatment, the value of AST was at the control level, whereas ALT was 1.4-fold higher and triglycerides were increased twofold.

Non-enzymatic protein glycation was confirmed by the examination of the glycation levels of serum proteins. Compared with control rats, in the diabetic group, these were increased 2.5-fold. Treatment with LA lowered the level of glycation 1.6-fold. The level of GlyHb was twofold higher under diabetic conditions and was lowered after the treatment with LA to a level 1.2-fold above the control group. The percent of MetHb was almost tenfold higher in the diabetic group, while the LA treatment of diabetic rats reduced the level of MetHb threefold in comparison with the control group. Hb was decreased in diabetic rats 1.3-fold compared with the control group, and it increased after the LA treatment of diabetic animals (Table 1).

RBC aggregation is an important property that influences changes in viscosity that affects blood flow. It is significantly altered by hyperglycemia [7]. To assess the effect of LA administration on RBC aggregation, we examined the changes in concentrations of α_2M and albumin, two plasma proteins that contribute to the aggregation process. Namely, increased α_2M and decreased albumin concentrations are in positive correlation with RBC aggregation [42]. In diabetic rats, serum α_2M was increased about 40-fold, whereas the treatment with LA lowered the α_2M to the 2.3-fold increase above the control level (Fig. 1). In diabetic rats, serum albumin decreased 1.6-fold below the control level, while the LA treatment restored the albumin level to the 1.5-fold increase measured in the diabetic group. The treatment of non-diabetic rats with LA did not cause significant changes in any of the examined parameters when compared with the control group.

Total iron and ferrous iron serum content and antioxidant potential of LA in the serum of control and diabetic animals

LA provides antioxidant activity through ROS scavenging, by chelating Fe^{2+} and Cu^{2+} and its ability to regenerate endogenous antioxidants [43]. We observed that the reducing property of LA and its ability to chelate Fe^{2+} are dose dependent (Fig. 2). In the serum of diabetic rats, the total iron content was increased by about 30%, while the LA treatment reduced the total iron level to about 80% of the level measured in the intact control. In rats that were only administered LA, no significant change in total iron content was observed (Fig. 2). The Fe^{2+} level increased threefold in diabetic rats, while the LA treatment lowered it to about 60% of the level observed in the intact control. In the serum of the LA-treated control rats, no significant change in the Fe^{2+} level was detected (Fig. 2). The antioxidant potential of LA in the serum of all four experimental groups was evaluated by the DPPH assay (Fig. 2). The inhibition of DPPH after the treatment of diabetic animals with LA was sevenfold higher compared with the control groups. The level of the free radical–scavenging capability in the serum of non-diabetic rats treated with LA was 12-fold higher compared with the control.

Parameters of oxidative stress in RBC

The redox status in RBC of each experimental group was distinguished by the levels of the basic biomarkers of

Table 1 The biochemical parameters in untreated and LA-treated non-diabetic and STZ-treated diabetic rats

	Experimental groups			
	NDM (non-diabetic)	DM (diabetic)	NDM + LA (non-diabetic + LA)	DM + LA (diabetic + LA)
N	7	8	7	8
Glucose (mmol/L)	5.8 ± 0.3^{a}	43.8 ± 2.3^{b}	4.3 ± 0.2^{a}	19.9 ± 1.2^{c}
Glycation (A)	0.47 ± 0.03^{a}	1.05 ± 0.05^{b}	0.44 ± 0.02^{a}	$0{,}77 \pm 0.04^{c}$
Hb (g/100 mL)	7.1 ± 0.4^{a}	5.5 ± 0.3^{b}	6.9 ± 0.4^{a}	6.5 ± 0.3^{c}
GlyHb (μmol fructose/g Hb)	9.8 ± 0.5^{a}	18.5 ± 0.9^{b}	9.6 ± 0.5^{a}	11.9 ± 0.6^{c}
MetHb (%Hb)	1.21 ± 0.06^{a}	11.73 ± 0.55^{b}	1.15 ± 0.05^{a}	3.6 ± 0.16^{c}
AST (U/L)	136 ± 5.1^{a}	262 ± 11.1^{b}	140 ± 6.1^{a}	147 ± 7.4^{a}
ALT (U/L)	52 ± 2.3^{a}	161 ± 7.5^{b}	58 ± 2.1^{a}	74 ± 2.8^{c}
Triglycerides (mmol/L)	0.85 ± 0.05^{a}	4.62 ± 0.21^{b}	0.91 ± 0.05^{a}	1.75 ± 0.09^{c}

Values are means ± S.E.M. for the indicated number of rats in each group (N)

Values not sharing a common superscript letter differ significantly at $P < 0.05$ (DMRT)

N number of rats per group, *Hb* hemoglobin, *GlyHb* glycated Hb, *MetHb* methemoglobin, *AST* alanine aminotransferases, *ALT* aspartate aminotransferases

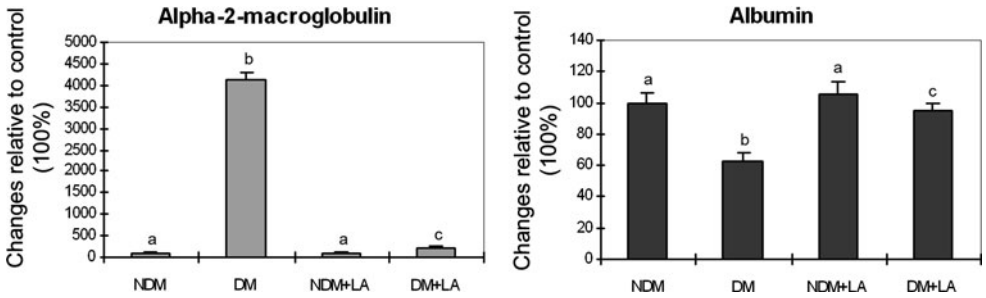

Fig. 1 The effect of LA treatment on the levels of α_2-macroglobulin and albumin in the serum of control and diabetic rats. *NDM* non-diabetic control; *DM* untreated diabetic; *NDM + LA* LA-treated NDM; *DM + LA* LA-treated DM. Values are means ± S.E.M. Means not sharing a *common letter* are significantly different between groups ($P < 0.05$)

oxidative stress: lipid peroxidation, free intracellular thiols, total and reduced GSH and GSSP (Fig. 3). The level of TBARS in RBC served as an indicator of lipid peroxidation. TBARS were increased twofold in the serum of diabetic rats. The treatment with LA reduced lipid peroxidation to the control level. Treatment of non-diabetic rats with LA did not significantly change the TBARS levels. Free SH groups are vital in cellular defenses against endogenous and exogenous oxidants [44]. The levels of free intracellular thiols in RBC are shown in Fig. 3. A threefold decrease in the SH content was observed in RBC from diabetic rats. This was restored to a value that was 1.6-fold lower than in control rats after the LA treatment. The SH content of RBC in the LA-treated control group did not change significantly from the intact control. GSH is a major SH antioxidant [45]. Reduced GSH in RBC in the diabetic group was 1.5-fold lower when compared with the control, while the treatment with LA restored the physiological level of GSH. The administration of LA to non-diabetic rats had no effect on GSH (Fig. 3). Oxidative stress can also result in the formation of mixed disulfides between cysteinyl residues of proteins and GSH to form GSSP that are directly linked to the redox status of the cell [46]. The GSSP level in RBC isolated from diabetic rats was threefold higher than that from the control level. The treatment of diabetic rats with LA lowered the GSSP level to 1.4-fold above the control level. In RBC from the LA-treated control group, no change in GSSP level relative to the intact control was observed.

Antioxidative enzymes in RBC

Disturbances of the antioxidant defense in diabetes comprise alterations in the activities of antioxidant enzymes [47]. The activities of the antioxidative enzymes in RBC in all of the examined experimental groups are presented in Fig. 4. In diabetic rats, CuSOD activity decreased about 25% compared with the non-diabetic group. LA treatment of diabetic rats restored CuSOD activity to 90% of the level measured in the non-diabetic control. CuSOD activity in the LA-treated non-diabetic rats was not affected significantly compared with the intact control. Similar results

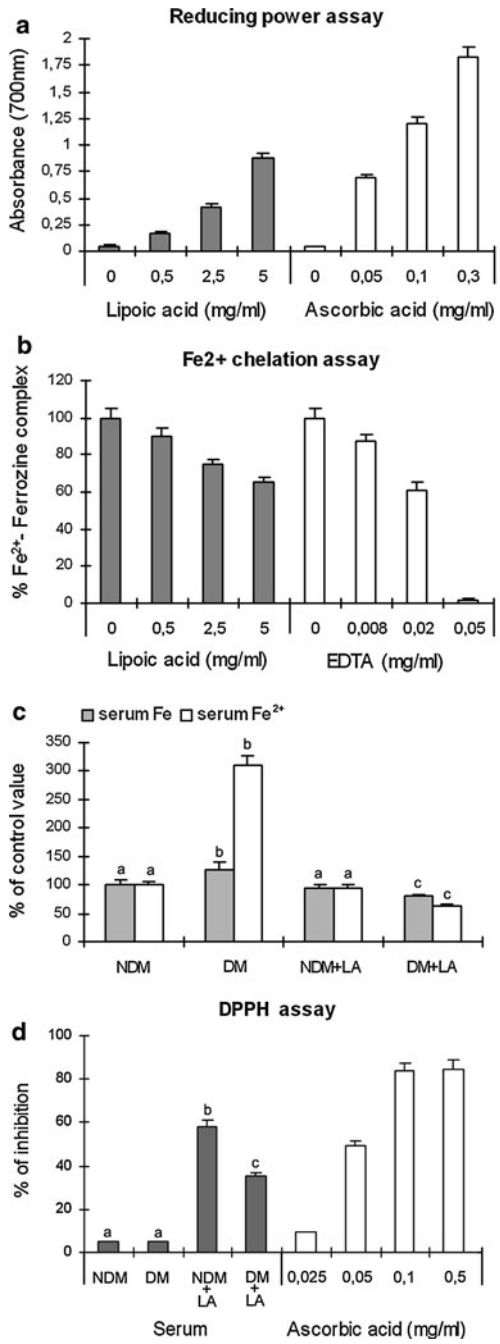

Fig. 2 In vitro antioxidant activity of LA (**a**, **b**), the serum iron content (**c**) and antioxidant potential of LA in the serum (**d**) of control and diabetic rats. Total reductive potential (**a**) and Fe^{2+} chelation ability (**b**) of different concentrations of LA. Ascorbic acid and EDTA were used as standards. The data are expressed as the percentage inhibition of chromogen formation. The results of the assays are presented as the mean ± S.E.M. from six parallel measurements. Changes in total and free chelatable iron levels in the serum of control and diabetic rats (**c**). DPPH activity in the serum of all four experimental groups. Ascorbic acid was used as a standard (**d**). *NDM* non-diabetic control; *DM* untreated diabetic; *NDM + LA* LA-treated NDM; *DM + LA* LA-treated DM. Values are the mean ± S.E.M for the indicated number of animals (N) in each group (see Table 1): Means that do not share a *common letter* are significantly different between groups ($P < 0.05$)

were also obtained for CAT activity. A 30% decrease in CAT activity was measured in RBC from diabetic rats compared with the non-diabetic group. The LA treatment of diabetic rats normalized CAT activity to 90% of the basal value. CAT activity in RBC from the LA-treated control group was at the level of the intact control. Diabetes induced a 2.2-fold decrease in GST activity, while in RBC of the LA-treated diabetic animals it was about 1.3-fold lower than the control level. In RBC from the LA-treated control group, GST activity was 1.1-fold than in the non-treated control.

O-GlcNac modifications in RBC

The most frequent type of intracellular enzymatic glycosylation is O-linkage of N-acetylglucosamine (O-GlcNAc) that plays a key role in glucose toxicity [48]. Examination of the hemolysates of all four examined experimental groups by SDS-PAGE did not reveal significant qualitative or quantitative differences between the samples (Fig. 5a). Equal amounts of hemolysates were passed through a wheat germ agglutinin (WGA) column, and the glycosylated proteins were purified with 0.4 M and 0.5 M N-acetylglucosamine. Subsequent examination by SDS-PAGE revealed qualitative and quantitative differences (Fig. 5b). Under diabetic conditions, the number and amount of O-GlcNAc-modified proteins were increased, while the LA treatment reduced the level of O-GlcNAc-modified proteins. Electrophoresis of proteins obtained from control and LA-treated non-diabetic animals did not reveal any differences between the respective protein profiles (Fig. 5b). The proteins present in the hemolysates and the fractionated, O-GlcNAc-containing proteins were subjected to immunoblot analysis with anti-CuZnSOD, anti-CAT, anti-HSP90 and anti-HSP70 antibodies. Immunoblot analysis of total RBC hemolysates did not reveal changes in the expression profiles of CuZnSOD, CAT and HSP70 in any of the experimental groups (Fig. 5c). Only HSP90 was considerably induced in diabetic animals. An even higher HSP90 protein level was observed in diabetic animals that were treated with LA. In hemolysates from control animals, HSP90 was present in traces, while in the LA-treated non-diabetic animals, HSP90 was slightly increased (Fig. 5c). O-GlcNAc-modified antioxidant enzymes CuZnSOD and CAT were detected in the diabetic group, while the LA treatment partially prevented O-GlcNAc glycosylation of CuZnSOD and CAT (Fig. 5d). Both HSP proteins were O-GlcNAc modified in the diabetic group (Fig. 5d). O-GlcNAc-modified HSP90 was detected in trace amounts compared with HSP70. The LA treatment of diabetic rats reduced the level of O-GlcNAc-modified HSP70 and completely prevented the O-GlcNAc modification of HSP90 (Fig. 5d).

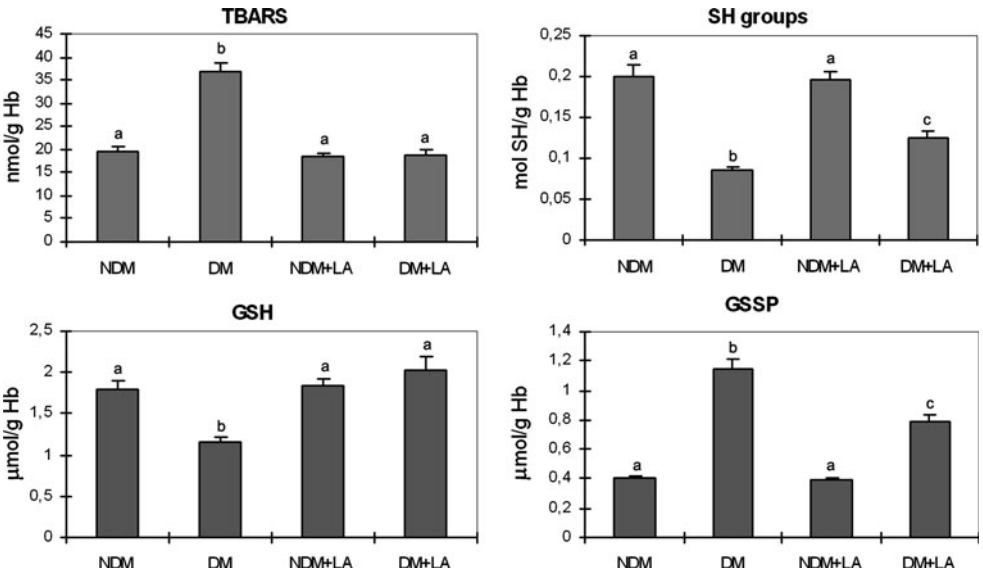

Fig. 3 The effect of LA treatment on lipid peroxidation (TBARS), the content of free sulfhydryl groups (SH), the level of reduced glutathione (GSH) and glutathionylated proteins (GSSP) in hemolysates of non-diabetic and STZ-induced diabetic rats. The values are presented as the mean ± S.E.M for the indicated number of animals (N) in each group (see Table 1): *NDM* non-diabetic control; *DM* untreated diabetic; *NDM + LA* LA-treated NDM; *DM + LA* LA-treated DM group. Means that do not share a *common letter* are significantly different between groups ($P < 0.05$)

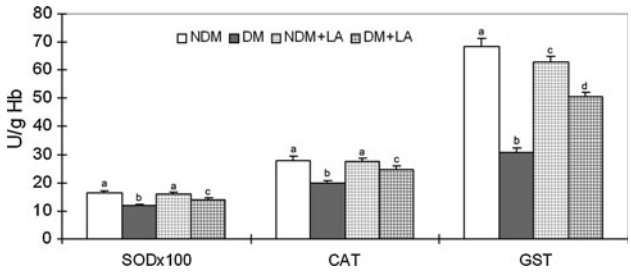

Fig. 4 The effect of the LA treatment on the activities of superoxide dismutase (SOD), catalase (CAT) and glutathione-S-transferase (GST) in hemolysates of non-diabetic and STZ-induced diabetic rats. Values are the mean ± S.E.M for the indicated number of animals (N) for each group (see Table 1): *NDM* non-diabetic control; *DM* untreated diabetic; *NDM + LA* LA-treated NDM; *DM + LA* LA-treated DM group. Means that do not share a *common letter* are significantly different between groups ($P < 0.05$)

Discussion

In this work, we showed that the administration of LA to diabetic rats plays a beneficial role by preserving the structural and functional integrity of RBC by adjusting the redox disturbance and by decreasing O-GlcNAc modifications of antioxidant enzymes and heat shock proteins. The improvement of the biochemical parameters of diabetes, such as glucose levels, and ALT and AST, the indicators of liver, kidney, heart, muscle damage that was observed after the treatment with LA led us to conclude that LA exerts an important beneficial systemic effect. While a number of therapeutic approaches are based on the effects of LA in both the prevention and treatment of diabetes-induced oxidative stress, the effect of LA treatment at doses that produce a significant reduction of oxidative stress has not been clearly demonstrated in the prevention of diabetic complications [49].

RBC are highly specialized cells whose main functions are oxygen transport and the mediation of carbon dioxide transport [50]. They are more susceptible to high glucose levels and diabetes-induced oxidative stress compared with other tissues due to their high content of iron and polyunsaturated fatty acids and role as oxygen transporters. Increased glucose levels affect basic RBC functions [7]. The biochemical alterations in plasma and RBC in diabetes affect their hemorrheologic properties such as aggregation and deformability. Increased concentrations of plasma fibrinogen and α_2M and decreased albumin concentration contribute to RBC aggregation [42, 51]. The present study revealed that the considerable increase in α_2M and decrease in albumin observed in diabetic rats were significantly reduced after the LA treatment. Turecký and co-workers [52] showed that α_2M levels are significantly elevated in type 1 diabetic patients who exhibit complications, than in diabetic patients without complications. The authors pointed out that elevated α_2M levels in diabetes contribute to the development of diabetic angiopathy.

The results obtained from the DPPH assay of rat serum show that LA can effectively inhibit oxidative stress in vivo. The increased level of MDA as a typical by-product of lipid peroxidation is considered to be a reliable marker of oxidative stress and RBC functionality [53], since lipid degradation affects membrane integrity and cell functionality. Judging by its effect on the MDA level, the results obtained in this study show that the LA treatment of diabetic rats almost completely prevented the RBC lipid peroxidation and presumably preserved membrane integrity. The antioxidant effects of LA are based on LA interaction with peroxyl radicals that initiate lipid peroxidation [43, 54].

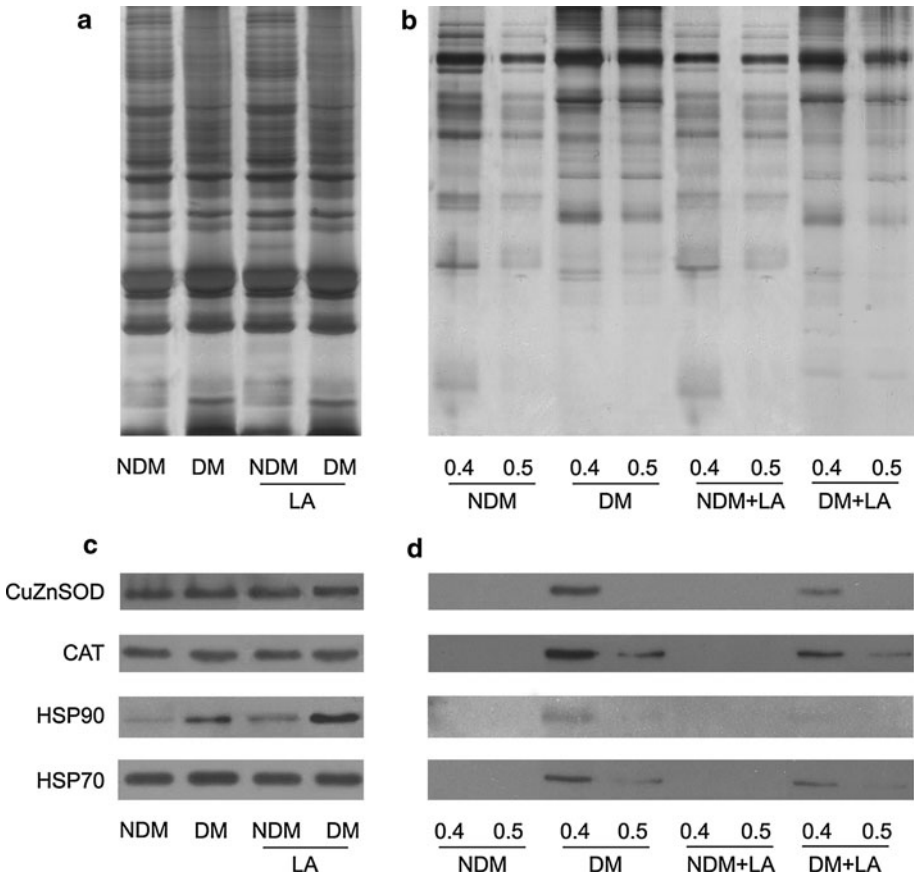

Fig. 5 Silver staining of hemolysates (**a**) and glycosylated proteins from hemolysates purified with 0.4 M and 0.5 M N-acetylglucosamine (**b**) of control and diabetic rats. Immunoblot analysis with anti-CuZnSOD, anti-CAT, anti-HSP90 and anti-HSP70 antibodies of hemolysates (**c**) and glycosylated proteins from hemolysates purified with 0.4 M and 0.5 M N-acetylglucosamine (**d**) of control and diabetic rats. *NDM* non-diabetic control; *DM* untreated diabetic; *NDM + LA* LA-treated NDM; *DM + LA* LA-treated DM

RBC are a major component of the antioxidant capacity of the blood, which is expressed through the glutathione system, the antioxidant enzyme system, as well as low-molecular-weight antioxidants associated with the erythrocyte membrane [9, 13]. The results of this study are in agreement with other reports that have documented decreased activities of erythrocyte CAT and SOD in STZ-induced diabetic rats [55, 56]. We also observed that GST activity was decreased in diabetic rats. This is in agreement with Anwar and Meki [57] who reported that GST activity in RBC declines in diabetes. We showed that the LA treatment of diabetic animals improved the capability of RBC to counteract oxidative stress by normalizing SOD, CAT and GST enzymatic activities. Recent reports [58] have shown that LA synergizes antioxidant activities by acting as a strong chain-breaking antioxidant.

In addition to the antioxidant enzymes, the GSH system also contributes to the regulation of redox homeostasis. The presented results revealed that the LA treatment restored the total protein SH content in RBC of diabetic rats, which allowed for the observed increased GSH levels. Post-translational *S*-glutathionylation leads to the addition of GSH in the form of a tripeptide to protein cysteine residues, resulting in the formation of glutathione-bound proteins [46]. This process is promoted by oxidative stress and is activated to protect specific protein SH groups from harmful modifications [59], thereby preserving the GSH in GSSP [60] and modulating the activities of cellular enzymes [61, 62]. We reported that the high GSSP level in RBC from diabetic rats was significantly reduced after LA administration, suggesting that LA protects RBC from oxidative stress. Normal functioning of the response to oxidative stress in RBC (the appropriate activation of antioxidant enzymes and the GSH system) is important for normal functioning of RBC. Thus, defective and/or unbalanced antioxidant defenses have been implicated in hemolysis [60, 62].

The main outcome of RBC hemolysis is iron release from Hb. The Hb levels in LA-treated diabetic rats exhibited very slight fluctuations from the basal level, pointing to a prominent role of LA in suppressing hemolysis. As the amount of Hb is directly proportional to the percentage of hemolysed RBC, it is considered a prominent marker of RBC viability. The toxicity of iron arises from its ability to catalyze the formation of oxygen-derived free radicals that can interact with cellular membranes and cytoplasmic constituents [63, 64]. Our results show that the LA treatment lowered the increased iron and ferrous ion levels in diabetic rats. It has been shown that LA provides antioxidant activity by chelating Fe^{2+} and Cu^{2+} ions [65].

The metal chelating activity of LA was confirmed in the present report. Raised metHb levels are also the consequence of oxidative stress under diabetic conditions [66]. Oxidized MetHb was significantly lowered in RBC from diabetic rats treated with LA due to its in vitro reducing capability. Our results are in agreement with the in vitro experiments of Coleman and co-workers [67] who reported on the significant reduction in sulphone metabolite-mediated MetHb in diabetic and non-diabetic RBC during 2 h after treatment with LA.

High glucose concentrations promote toxicity, not only through the formation of ROS and the reduction of the effectiveness of the cell's antioxidant defenses, but also through non-enzymatic glycosylation reactions [68, 69]. In uncontrolled diabetic conditions, an increased number of glycated proteins, including Hb, are observed [70]. In our study, the treatment with LA reduced the glycation of both serum proteins and Hb. This result is in correlation with in vitro studies that showed that GlyHb levels were higher in RBC that were maintained in the presence of 50 mmol/L glucose than in RBC maintained in the presence of 5 mmol/L glucose. The increase in GlyHb was blocked significantly when RBC were pretreated with LA [71]. The authors also showed that the level of GlyHb in RBC that were incubated in the presence of MDA was increased significantly compared with RBC that were incubated with glucose alone. This result points to the role of MDA in promoting Hb glycation [71]. Therefore, LA probably alleviates Hb glycation also by lowering the MDA levels. Moreover, there is also a negative correlation between GSH and GlyHb in diabetic patients, confirming the link between hyperglycemic conditions and GSH depletion [72]. High glucose concentrations deplete GSH through the polyol pathway as a result of increased consumption of NADPH that is required for GSH regeneration.

Aside from the non-enzymatic glycation of proteins, under diabetic conditions, nuclear and cytoplasmic proteins also undergo increased enzymatic glycosylation—O-GlcNAc-modification [73]. The attachment of O-GlcNAc is catalyzed by O-GlcNAc transferase (OGT), while β-N-acetyl-glucosaminidase (O-GlcNAcase) removes the O-GlcNAc group. Excessive O-GlcNAcylation is a reflection of a prolonged hyperglycemic state, and the extent of O-GlcNAcylation of nuclear and cytoplasmic proteins is highly sensitive to the concentrations of glucose [74]. Although RBC are among the simplest cells, recent analysis of the RBC proteome has revealed the occurrence of more complex cellular processes in RBC, including O-GlcNAc protein modifications [75]. The results in this study showed increased levels of O-GlcNAc-modified antioxidant enzymes CuZnSOD and CAT, as well as HSP70 and HSP90 under diabetic conditions, and a significant reduction of their O-GlcNAc modification following the LA treatment. We observed that in diabetic rats that the decreased enzyme activities of CuZnSOD and CAT were accompanied by unchanged protein levels. The enzymatic silencing of CuZnSOD and CAT could be the result of their post-translational modifications, including O-GlcNAcylation. In vitro studies have shown that glycosylation causes a 40% lowering of CuZnSOD activity in RBC [76].

HSP function primarily as molecular chaperones that prevent protein aggregation, facilitate the folding of other cellular proteins and target improperly folded proteins to specific pathways for degradation [15]. Defects in HSP functions could contribute to the complications in diabetes that generally interfere with proper HSP synthesis and responses [77]. LA induces HSP60 in rat heart, possibly by activating HSF-1 via increased disulfide formation in target proteins [78]. In a study of type 1 diabetic patients with neuropathy, Strokov and co-workers [79] observed that LA administration restored HSP levels and NO production and improved neuropathic symptoms. We observed that the LA treatment slightly induced HSP90 protein expression in RBC when administrated to the control animals while boosting HSP90 protein expression under diabetic conditions. Also, the LA treatment decreased the levels of O-GlcNAc-modified HSP70 and HSP90 in diabetic animals. In RBC, transcriptional regulation occurs prior to enucleation [12]. After immunoblot analysis of proteins prepared from RBC of control and diabetic rats with anti-lamin B antibody, we observed the same levels of lamin B, one of the major protein constituents of the peripheral nuclear lamina (data not shown). Based on this result, we hypothesized that the LA treatment induced an increase in HSP90 in nucleated reticulocytes. The induction of HSP90 and the lower levels of O-GlcNAc-modified HSP70 and HSP90 as a result of the LA treatment is an important defense mechanism in RBC. Since RBC cannot replace their damaged proteins, these chaperones monitor, protect and maintain the structure and stability of erythrocyte proteins.

Disordered metabolism and homeostasis in RBC affect the antioxidant potential of the whole body. The results presented in this study show that the treatment of diabetic rats with LA diminished lipid peroxidation, the glycation of serum proteins and Hb, improved the activity of the antioxidant enzymes and balanced the GSH system. By reducing the level of O-GlcNAc-modified antioxidant enzymes in RBC and inducing the synthesis of HSP90, LA preserves the structural and functional integrity of RBC. Functional and healthy RBC could delay or inhibit further diabetic complications, especially neuropathy.

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