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Decreased *O*-GlcNAcylation of the key proteins in kinase and redox signalling pathways is a novel mechanism of the beneficial effect of α-lipoic acid in diabetic liver

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Abstract

The present study aimed to investigate the effects of the treatment with α-lipoic acid (LA), a naturally occurring compound possessing antioxidant activity, on liver oxidant stress in a rat model of streptozotocin (STZ)-induced diabetes by examining potential mechanistic points that influence changes in the expression of antioxidant enzymes such as catalase (CAT) and CuZn/Mn superoxide dismutase(s) (SOD). LA was administered for 4 weeks by daily intraperitoneal injections (10 mg/kg) to STZ-induced diabetic rats, starting from the last STZ treatment. LA administration practically normalised the activities of the indicators of hepatocellular injury, alanine and aspartate aminotransferases, and lowered oxidative stress, as observed by the thiobarbituric acid-reactive substance assay, restored the reduced glutathione:glutathione disulphide ratio and increased the protein sulfhydryl group content. The lower level of DNA damage detected by the comet assay revealed that LA reduced cytotoxic signalling, exerting a hepatoprotective effect. The LA-treated diabetic rats displayed restored specific enzymatic activities of CAT, CuZnSOD and MnSOD. Quantitative real-time PCR analysis showed that LA restored *CAT* gene expression to its physiological level and increased *CuZnSOD* gene expression, but the gene expression of *MnSOD* remained at the diabetic level. Although the amounts of CAT and CuZnSOD protein expression returned to the control levels, the protein expression of MnSOD was elevated. These results suggested that LA administration affected CAT and CuZnSOD expression mainly at the transcriptional level, and MnSOD expression at the post-transcriptional level. The observed LA-promoted decrease in the *O*-GlcNAcylation of extracellular signal-regulated kinase, protein 38 kinase, NF-κB, CCAAT/enhancer-binding protein and the antioxidative enzymes themselves in diabetic rats suggests that the regulatory mechanisms that supported the changes in antioxidative enzyme expression were also influenced by post-translational mechanisms.

Key words: α-Lipoic acid: Diabetic liver: *O*-GlcNAcylation: Oxidative stress

Oxidative stress appears at the early onset of diabetes mellitus and then increases progressively. It is generally accepted that oxidative stress is involved in the development of diabetic complications in diverse tissues[1]. The liver is the main organ of detoxifying and oxidative processes, and at the early stage of experimental streptozotocin (STZ)-induced diabetes, biomarkers of oxidative stress are elevated in the liver[2]. Recently, a direct link has been described between diabetic oxidative stress and the pathogenic sequence of events leading to hepatocellular carcinoma and non-alcoholic steatohepatitis in diabetes[3]. The importance of liver oxidative stress is further highlighted by experimental and clinical data, suggesting that oxidative stress-mediated damage to the liver tissue underlies most of the pathological alterations in liver morphology and function observed in diabetes[4,5]. Diabetes is estimated to be the most common cause of liver disease[6], which has in turn emerged as a major cause of death[3].

Hyperglycaemia, the hallmark manifestation of diabetes, promotes the formation of elevated levels of free radicals and oxidative stress via different routes of activation: polyol pathway; protein kinase C activation; increase in hexosamine flow rate; advanced glycation end-product signalling through

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; C/EBPβ, CCAAT/enhancer-binding protein; CAT, catalase; ERK, extracellular signal-regulated kinase; GSH, reduced glutathione; GSSG, glutathione disulphide; LA, α-lipoic acid; MDA, malondialdehyde; *O*-GlcNAc, *O*-linked *N*-acetylglucosamine; p38, protein 38; p65, protein 65; ROS, reactive oxygen species; SH, sulfhydryl; SOD, superoxide dismutase; STZ, streptozotocin.

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the receptor for advanced glycation end products. Hyperglycaemia also exerts an important effect by contributing to increased enzymatic post-translational glycosylation of proteins with *O*-linked *N*-acetylglucosamine (*O*-GlcNAc) groups. *O*-GlcNAcylation has an important function in adjusting cellular activity to glucose availability. It is a dynamic and reversible post-translational modification analogous to phosphorylation, which affects normal protein functions by altering their enzymatic activities[7], protein–protein interactions[8], DNA binding[9], subcellular localisation and proteolytic processing[10]. In diabetes, increased enzymatic post-translational *O*-GlcNAcylation of proteins is associated with the adverse effects of hyperglycaemia, and is an established pathogenic contributor to glucose toxicity and insulin resistance[11,12].

Oxidative stress is caused by the cell's inability to detoxify generated reactive oxygen species (ROS) by an interacting network of several constitutively expressed antioxidant enzymes and non-enzymatic antioxidant components. The principal antioxidant enzymes and non-enzymatic components are superoxide dismutases (SOD), catalases (CAT), as well as small molecules such as glutathione (reduced glutathione (GSH)), vitamins C and E, and other compounds. The mechanism of signal transduction from elevated ROS levels that leads to the activation of the expression of detoxifying/defensive genes includes complex kinase pathways (protein 38 (p38) mitogen-activated protein kinase and extracellular signal-regulated kinase (ERK)) involved in the activation of NF-κB protein 65 (p65)[13], and the liver-enriched transcription factors CCAAT/enhancer-binding protein β (C/EBPβ)[14]. The resulting activation of transcription factors and their binding to a common *cis*-element, the antioxidant response element, up-regulates the transcription of many defensive genes[15], notably the genes encoding for CuZnSOD, MnSOD and CAT[16].

As oxidative stress has been shown to be important in the pathogenesis of liver disease in diabetes, strategies to reduce the formation of ROS have been considered for diabetes management[17]. While pilot studies with vitamin E have been conducted with promising results, a meta-analysis of high-dose vitamin E revealed an increase in overall mortality[3], emphasising the need to examine other antioxidant compounds. α-Lipoic acid (LA), an endogenously synthesised compound available as a nutritional supplement, has gained considerable attention as a powerful antioxidant[18]. LA is an organosulphur molecule derived from octanoic acid, which functions as a co-enzyme in pyruvate dehydrogenase and α-ketoglutarate dehydrogenase mitochondrial reactions leading to ATP production. LA possesses direct radical-scavenging and metal-chelating properties and has the ability to regenerate other antioxidants. LA is a clinically accepted therapeutic agent used in the treatment of diabetic neuropathy[19]. Among its observed beneficial properties in diabetes management[20], LA has been reported to exert positive effects on antioxidative enzyme activities in experimentally induced diabetic rat liver[21].

Given that the diabetic liver is susceptible to ROS-mediated injury, we investigated the effects of LA treatment on liver oxidative stress and on the events potentially involved in the regulation of the expression of the major antioxidant enzymes CuZnSOD, MnSOD and CAT in an experimental model of STZ-induced diabetes. We examined the effects of LA administration to diabetic rats on specific enzymatic activities, protein and mRNA levels, and post-translational *O*-GlcNAcylation of CuZnSOD, MnSOD and CAT in the liver. We also assessed the changes in protein levels and the glycosylation status of ERK and p38 kinase, and of the transcription factors NF-κB p65 and C/EBPβ.

Experimental methods

Animals and treatments

All experimental procedures involving animals were approved by the Committee for Ethical Animal Care and Use of the Institute for Biological Research, Belgrade, and are in accordance with the Guide for the Care and Use of Laboratory Animals, published by the US National Institutes of Health (NIH publication no. 85/23, revised in 1986).

Male albino (2·5-month-old) rats of the Wistar strain were used. Diabetes was induced by the administration of multiple low doses of STZ (MP Biomedicals). STZ was dissolved in freshly prepared sodium citrate buffer (0·1 M, pH 4·5) and injected intraperitoneally at a dose of 40 mg/kg per d for five consecutive days[22]. The control rats were injected with the vehicle alone (citrate buffer). Rats were considered as diabetic when their blood glucose concentration was >20 mmol/l. Blood glucose was measured (Accu-Chek Active) 24 h after the last STZ injection. The rats were divided into four experimental groups: control group (n 7); diabetic group (n 8); control group treated with LA (n 7); diabetic group treated with LA (n 8). Freshly prepared LA (Alphalipoin; Ivančić i sinovi) was intraperitoneally injected (10 mg/kg per d) daily[23] for 4 weeks into the control and diabetic rats, starting from the last day of the STZ treatment. The body weights of all rats were recorded on the first and the last day of the experiment. All the animals were killed 4 weeks after diabetes induction.

Biochemical analysis

Serum was prepared after collecting the blood into coagulant-coated tubes followed by centrifugation (2000 ***g***, 10 min). Glucose concentrations were determined in serum with a commercial kit (Gluco-quant Glucose/HK; Boehringer). Serum TAG were measured by the GPO-PAP method (Randox Laboratories). Activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were estimated in serum by measuring oxaloacetate and pyruvate production, respectively, using an optimised standard UV kinetic method kit (GPT (ALAT) IFCC mod; GOT (ASAT) IFCC mod). Glycation of serum proteins was measured by the fructosamine assay as described previously[24]. 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. The samples were incubated (1 h, 37°C), and absorbance was measured at 595 nm.

Lipid peroxidation assay

The level of lipid peroxidation was estimated by measuring the concentration of the reactive by-product malondialdehyde

(MDA) in the thiobarbituric acid-reactive substance assay as described previously[25]. Fresh liver tissue was homogenised in 1·15 M-KCl (100 mg/0·9 ml). An aliquot of the homogenate (0·1 ml) was mixed with 0·2 ml of 8·1 % SDS, 1·5 ml of 20 % CH_3COOH (pH 3·5), 1·5 ml of 0·8 % thiobarbituric acid and 0·7 ml of water and then incubated (95°C, 60 min). After cooling the mixture to room temperature, 1 ml of water and 5 ml of *n*-butanol–pyridine (15:1, v/v) were added, mixed and centrifuged at 3000 ***g*** for 10 min. The absorbance of the supernatant was measured at 532 nm and used for calculating the MDA content (nmol MDA/mg protein).

Measurement of reduced glutathione and reduced glutathione disulphide

GSH and glutathione disulphide (GSSG) concentrations were determined by the standard recycling method with the Glutathione Assay Kit (Cayman Chemicals Company). Fresh liver (200 mg) was homogenised in 180 ml of phosphate buffer (100 mM-NaH_2PO_4 and 1 mM-EDTA, pH 7·5) and then centrifuged (5000 ***g***, 5 min). The supernatant was deproteinised with 5 % 5-sulfosalicylic acid and then centrifuged (7000 ***g***, 5 min, 4°C). The resulting supernatant was neutralised with triethanolamine and stored at −80°C until use. For GSH determination, 50 μl of the deproteinised sample were incubated (30 min, room temperature) with 150 ml of the reaction mixture (0·1 M-sodium phosphate buffer (pH 7·5) containing 1 mM-EDTA, 0·3 mM-5,5'-dithio-bis(2-nitrobenzoic acid), 0·4 mM-NADPH and 1 U glutathione reductase I/ml). The reaction rate was monitored by measuring absorbance at 412 nm. GSH content was determined using a calibration curve established with a series of glutathione standard dilutions that were processed through exactly the same procedure as described for the samples. GSSG was quantified after derivatisation of GSH with 2-vinylpyridine so that only GSSG is recycled to GSH. In brief, 50 ml of the deproteinised sample were mixed with 2-vinylpyridine at a final concentration of 10 mM. Standard amounts of GSSG were treated in the same way. After incubation (1 h, room temperature), the samples and the standards were analysed as described above using a calibration curve. GSSG was subtracted from the total GSH to determine the actual GSH level and the GSH:GSSG ratio.

Determination of protein oxidation

Protein sulfhydryl (SH) groups were determined according to the Ellman method[26]. The isolated liver (0·1 g/ml) was minced and homogenised in sucrose buffer (0·25 M-sucrose, 1 mM-EDTA and 0·05 M-Tris–HCl, pH 7·4), sonicated and centrifuged (100 000 ***g***, 90 min) in a Beckmann rotor Ti 50. The liver homogenate (0·5 ml) was mixed with 0·1 M-phosphate buffer (pH 7·4, 0·5 ml) in a cuvette, followed by the addition of 0·2 ml of 3 mM-5,5-dithiobis (2-nitrobenzoic acid) and incubation for 10 min. Absorbance (*A*) was measured at 412 nm and the amount of SH groups was calculated according to the formula:

$$\text{mol SH/ml} = ((A_{\text{sample}}/14\,150) \times \text{dilution factor})/\text{ml}.$$

Alkaline comet assay

The comet assay was performed to estimate and compare the levels of DNA damage in the different experimental groups. A liver cell suspension was prepared by mincing tissue in ice-cold Harks buffered salt solution buffer (0·14 g $CaCl_2$/l, 0·4 g KCl/l, 0·06 g KH_2PO_4/l, 0·1 g $MgCl_2.6H_2O$/l, 0·1 g $MgSO_4.7H_2O$/l, 8·0 g NaCl/l, 0·35 g $NaHCO_3$/l, 0·09 g $Na_2HPO_4.7H_2O$/l and 1·0 g D-glucose/l) containing 20 mM-EDTA and 10 % dimethyl sulphoxide. The cell suspension (10 μl) was mixed with low-melting agarose onto a microscope slides and lysed for 2 h at 4°C in lysis buffer (2·5 M-NaCl, 100 mM-EDTA, 10 mM-Tris (pH 10) and 1 % Triton X-100). The slides were then incubated in electrophoresis buffer (300 mM-NaOH and 1 mM-EDTA, pH 13·0) for 30 min at 4°C and exposed to electrophoresis, after which they were placed in neutral buffer (0·4 M-Tris–HCl, pH 7·4) and stained with SYBR Green I (Sigma-Aldrich). DNA damage was quantified by measuring the displacement between the genetic material of the nucleus ('comet head') and the resulting 'comet tail'. Images were analysed with TriTekCometScore™ Freeware version 1.5 (http://www.AutoComet.com).

Antioxidant enzyme assays

Liver homogenates (0·1 g/ml) prepared in sucrose buffer (0·25 M-sucrose, 1 mM-EDTA and 0·05 M-Tris–HCl, pH 7·4) were sonicated and centrifuged (100 000 ***g***, 90 min) in a Beckmann rotor Ti 50. Protein concentrations in the homogenates were determined as described previously[27]. Total SOD activity was measured according to the epinephrine method[28] and expressed as U/mg protein. MnSOD activity was performed after pre-incubation with 8 mM-KCN. CuZnSOD activity was calculated from the difference between total SOD and MnSOD activities. CAT activity was measured by the rate of H_2O_2 decomposition and expressed as μmol-H_2O_2/min per mg protein[29].

Preparations of different cellular fractions

Nuclear and cytosol proteins were prepared from the liver tissue (100 mg/sample) using the ProteoJET Cytoplasmic and Nuclear Protein Extraction Kit (Fermentas), according to the manufacturer's instructions. Mitochondria were isolated by differential centrifugation[30].

Detection of O-linked N-acetylglucosamine protein modification

Proteins containing *N*-acetylglucosamine residues were isolated from liver homogenates. Liver homogenates were prepared as follows: liver was excised, finely cut and homogenised in a hand-held glass homogeniser in 0·25 M-sucrose, 1 mM-EDTA and 0·05 M-Tris–HCl (pH 7·4), followed by sonication and centrifugation (100 000 ***g***, 90 min) in a Beckmann rotor Ti 50. Equal amounts of the liver homogenates (1·5 mg) were passed through a column of wheat-germ lectin immobilised on Sepharose (Pharmacia) at 4°C[31].

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The column was pre-washed with 50 ml 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-dithiothreitol) containing 0·1 M-KCl. Glycosylated proteins were eluted with Z-buffer containing 0·1 M-KCl and *N*-acetylglucosamine (0·4 and 0·5 M). Equal aliquots of the homogenates and eluted proteins were separated by 12% SDS–PAGE[32]. Gels were silver-stained or were electrotransferred onto polyvinylidene difluoride membranes (Hybond-P; Amersham Pharmacia Biotech) and analysed by immunoblotting.

Western blot analysis

Liver homogenates and nuclear, cytosol or mitochondrial proteins (20 μg) were separated by SDS–PAGE, transferred onto polyvinylidene difluoride membranes and examined by immunoblot analysis[33] with polyclonal antibodies against rat MnSOD, CuZnSOD, ERK1/2, p38 MAPK, NF-κB p65, C/EBP((Santa Cruz Biotechnology) and CAT (Abcam). Detection was performed by an enhanced chemiluminescence detection system (Santa Cruz Biotechnology). Each polyvinylidene difluoride membrane was reprobed according to the supplier's protocol by incubation in a stripping solution (2 % SDS, 100 mM-β-mercaptoethanol and 62·5 mM-Tris–HCl, pH 6·8) for 35 min at 50°C. Quantification of immunoreactive bands was performed using TotalLab (Phoretix) electrophoresis software (version 1.10).

Real-time quantitative PCR analysis

Total RNA was isolated from the liver using the RNeasy Mini Kit (Qiagen). For complementary DNA synthesis, total RNA was treated with DNase I and reverse-transcribed with the RevertAid First Strand cDNA Synthesis Kit (Fermentas) using oligo(dT) primers. mRNA levels were quantified by real-time PCR on an ABI Prism 7000 Sequence Detection System (Applied Biosystems) using SYBR Green technology. The sequences of the used primers (Invitrogen) were as follows: 5′-CAG ATC ATG CAG CTG CAC CA-3′ and 5′-TCA GTC CAG GCT GAA GAG CA-3′ for forward and reverse primers of the rat *MnSOD* gene, respectively; 5′-GCA GAA GGC AAG CGG TGA AC-3′ and 5′-CGG CCA ATG ATG GAA TGC TC-3′ for forward and reverse primers of the rat *CuZnSOD* gene, respectively; 5′-GCG AAT GGA GAG GCA GTG TAC-3′ and 5′-GAG TGA CGT TGT CTT CAT TAG CAC TG-3′ for forward and reverse primers of the rat *CAT* gene, respectively. PCR were carried out in triplicate. Negative controls without templates were used in all PCR. Expression levels of the target genes were related to the averaged expression level of β-actin as a housekeeping gene.

Statistical analysis

Results are presented as means with their standard errors. Values of the parameters were normally distributed and data were analysed by parametric tests. Data presented in Fig. 2 (mRNA levels of *CuZnSOD*, *MnSOD* and *CAT*; protein levels of CuZnSOD and CAT in the cytosol and that of MnSOD in the mitochondria) were analysed by one-way ANOVA. Statistical significance was analysed by Duncan's multiple range test. The main effects of STZ-induced diabetes or LA treatment alone and in combination on the data presented in Table 1, Table 2 and Fig. 1 (comet assay) were analysed by two-way ANOVA, followed by the comparison of mean values using Duncan's test. Statistical significance was defined as $P<0·0001$.

Results

To objectively evaluate the effects of LA in diabetic rats, we attempted to identify in hepatic cells potential mechanistic points at the molecular level that are involved in the established antioxidant effects of LA.

Table 1. General and biochemical parameters of the control (C), diabetic (D) and α-lipoic acid (LA)-treated rats*
(Mean values with their standard errors from three experiments performed in triplicate)

	C		D		C+LA		D+LA	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Glucose (mmol/l)	5·8[a]	0·3	43·8[b]	2·3	4·3[a]	0·2	19·9[c]	1·2
TAG (mmol/l)	0·8[a]	0·05	4·6[b]	0·21	0·9[a]	0·05	1·75[c]	0·09
ALT (U/l)	52[a]	2	161[b]	7	58[a]	2	74[c]	3
AST (U/l)	136[a]	5	262[b]	11	140[a]	6	147[a]	7
Protein glycation *A*†	0·47[a]	0·03	1·05[b]	0·05	0·44[a]	0·02	0·77[c]	0·04
Body weight (g)	470[a]	20	185[b]	9	450[a]	19	275[c]	12
Liver weight (g)	12·8[a]	0·6	8·3[b]	0·4	11·9[a]	0·5	11·1[c]	0·6
Liver weight:body weight (%)	2·72[a]	0·13	4·48[b]	0·21	2·64[a]	0·12	4·03[c]	0·12
n	7		8		7		8	

ALT, alanine aminotransferase; AST, aspartate aminotransferase; C+LA, control rats treated with LA; D+LA, diabetic rats treated with LA.
[a,b,c] Mean values within a row with unlike superscript letters were significantly different ($P<0·0001$; Duncan's test).
* Data were analysed by two-way ANOVA (streptozotocin (STZ)-induced diabetes × LA treatment) followed by Duncan's multiple comparison test to compare the groups. This test was applied to all the analysed parameters when the significant difference observed by ANOVA was $P<0·05$. Two-way ANOVA revealed a significant effect of STZ-induced diabetes and LA treatment and the interaction between STZ-induced diabetes and LA treatment for all the tested parameters.
† Absorbance of nitro blue tetrazolium at 595 nm in fructosamine assay.

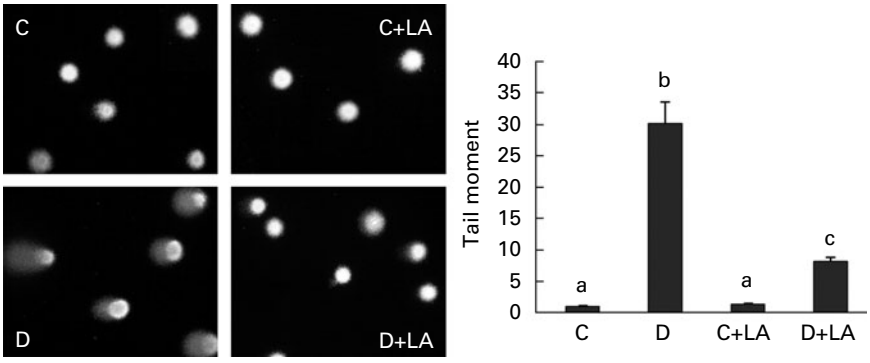

Fig. 1. Extent of DNA damage in the different experimental groups assessed by the comet assay. C, control rats; D, diabetic rats; C+LA, control rats treated with α-lipoic acid (LA); D+LA, diabetic rats treated with LA. Values are means from three experiments performed in triplicate, with their standard errors represented by vertical bars. Data were analysed by two-way ANOVA (streptozotocin-induced diabetes × LA treatment) followed by Duncan's test to compare the groups. [a,b,c] Mean values with unlike letters were significantly different ($P<0·0001$).

α-Lipoic acid administration improves general diabetes indicators

Compared with the normal rats, the STZ-treated animals presented the hallmark signs of diabetes (Table 1): elevated fasting blood glucose and serum TAG concentrations (8- and 6-fold, respectively); decline in body weight (61 %); increased serum protein glycation index (2-fold). The treatment of diabetic rats with LA significantly lowered fasting blood glucose and serum TAG concentrations (3- and 2-fold, respectively, relative to the control rats), improved the glycation index (13 % above the control value) and caused weight gain (an improvement by a 41·5 % reduction in the body weight of the control). LA administration to the control rats did not exert any significant effects on the examined diabetes indicators.

Assessment of the beneficial effects of α-lipoic acid administration on diabetic liver

Diabetic rats displayed the general signs of hepatocellular injury, manifested as increased specific activities of the liver enzymes ALT and AST (3- and 2-fold, respectively), a 35 % reduction in liver weight, and a sign of liver hypertrophy, revealing a 1·7-fold increase in the liver weight:body weight ratio (Table 1). Oxidative stress that accompanies the diabetic state is a confirmed pathogenic mechanism of organ dysfunction, resulting in different diabetic complications. Examination of the major parameters of oxidative stress revealed a 30 % increase in MDA concentration, measured using the thiobarbituric acid-reactive substance assay, a 43 % decrease in the GSH:GSSG ratio and 34 % lower concentrations of free thiol groups due to increased protein oxidation (Table 2). As expected, such a pro-oxidant environment in the liver established the conditions that promoted DNA damage, leading to the ultimate destructive consequence of severe oxidant stress. Next, we examined DNA damage in liver cells with the comet assay (Fig. 1). The comet tail moment (the product of the tail length and the fraction of DNA in the tail described by tail intensity) obtained served as a measure of DNA damage. A 30-fold higher tail moment in diabetic rats (Fig. 1) compared with the control rats revealed increased levels of DNA damage. These results present a compromised functional and structural integrity of diabetic rat liver cells.

Table 2. General markers of liver oxidative stress (lipid peroxidation (thiobarbituric acid-reactive substances; TBARS); glutathione oxidation (reduced glutathione (GSH):glutathione disulphide (GSSG)); protein oxidation (protein sulfhydryl (SH) groups); CuZn/Mn superoxide dismutase(s) (SOD) and catalase (CAT) activity)*

(Mean values with their standard errors from three experiments performed in triplicate)

	C		D		C+LA		D+LA	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
TBARS (nmol MDA/mg protein)	13·74[a]	0·69	17·81[b]	0·89	11·26[a]	0·53	14·6[c]	1·2
GSH:GSSG ratio	13·02[a]	0·62	7·39[b]	0·35	13·63[a]	0·65	13·93[a]	0·67
SH groups (mol SH/l)	0·94[a]	0·04	0·62[b]	0·02	0·95[a]	0·05	1·1[a]	0·06
tSOD (U/mg protein)	33·5[a]	1·65	27[b]	1·34	34·3[a]	1·71	33·2[a]	1·64
CuZnSOD (U/mg protein)	25·8[a]	1·23	20·5[b]	1·02	26·4[a]	1·32	25·7[a]	1·29
MnSOD (U/mg protein)	7·7[a]	0·54	6·5[b]	0·44	7·9[a]	0·55	7·5[a]	0·51
CAT (U/mg protein)	149·5[a]	7·3	58·35[b]	2·9	145[a]	7·25	142[a]	7·2

C, control rats; D, diabetic rats; LA, α-lipoic acid; C+LA, control rats treated with LA; D+LA, diabetic rats treated with LA; MDA, malondialdehyde; tSOD, total SOD.

[a,b,c] Mean values within a row with unlike superscript letters were significantly different ($P<0·0001$; Duncan's test).

* Data were analysed by two-way ANOVA (streptozotocin (STZ)-induced diabetes × LA treatment) followed by Duncan's multiple comparison test to compare the groups. This test was applied to all the analysed parameters when significant differences observed by ANOVA was $P<0·05$. Two-way ANOVA revealed a significant effect of STZ-induced diabetes and LA treatment and the interaction between STZ-induced diabetes and LA treatment for all the tested parameters.

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The administration of LA to the diabetic rats promoted an improvement of the examined parameters of oxidative stress in the liver, notably the complete restoration of the GSH:GSSG ratio and free thiol groups, and a significant lowering of MDA concentration (Table 2). The LA-promoted alleviation of oxidative stress reduced cytotoxic signalling, manifesting a decrease in DNA damage. In the LA-treated diabetic group, the tail moment was significantly reduced by 8-fold when compared with the control (Fig. 1). The LA-treated diabetic rats also exhibited the overall signs of improved liver function, almost normalised AST and ALT activities and a slight improvement in the liver weight:body weight ratio (Table 1). LA administration to the normal rats did not significantly affect the examined markers of oxidative stress or DNA damage.

Effect of α-lipoic acid administration on hepatic antioxidant enzyme status in diabetes

Oxidative stress is caused by the cell's inability to detoxify the generated ROS. In light of the observed improvement of diabetic oxidative stress in the rat liver after LA administration, we investigated the consequences of LA administration on hepatic antioxidant SOD and CAT enzyme activities (Table 2). The specific enzymatic activities of CAT and SOD were lower in diabetic rats. LA administration to the diabetic rats restored the specific activities of CAT, total SOD, CuZnSOD and MnSOD.

To examine the level of antioxidant enzyme expression controlled after LA administration, we analysed antioxidant gene transcription by quantitative real-time PCR and compared the results with relative changes in protein levels. In the liver of diabetic rats, the mRNA levels of *CAT*, *CuZnSOD* and *MnSOD* were reduced by 54, 32 and 28 %, respectively, in comparison with the controls (Fig. 2(A)). LA administration to the diabetic rats promoted the complete restoration of *CAT* gene expression to its physiological level (Fig. 2(A)). While *CuZnSOD* gene transcription was improved after LA administration to the diabetic rats, *MnSOD* transcription remained unchanged, at the level measured in the untreated diabetic rats (Fig. (2A)). The examination of liver subcellular fractions by immunoblot analysis (Fig. (2B)) showed that the protein levels of CAT and CuZnSOD were reduced by 20 and 25 % in the cytosol of diabetic rats, respectively. In contrast, the amount of MnSOD protein in the mitochondria was increased by 15·5 % in diabetic rats when compared with the control. The treatment of diabetic rats with LA restored the amounts of both cytosolic CAT and CuZnSOD to the control levels (Fig. 2(B)), while the amount of MnSOD protein in the mitochondria remained 8·5 % above the control level. The LA treatment of control animals did not elicit any changes in the mRNA and protein levels of the examined antioxidative enzymes.

Effect of α-lipoic acid administration on O-linked N-acetylglucosamine modification of antioxidative enzymes

Hyperglycaemia is associated with increased enzymatic post-translational *O*-GlcNAc modifications of diverse proteins, which influences different aspects of their functions. *O*-GlcN-Acylated proteins in liver homogenates were prepared using a wheat germ agglutinin column. Visual inspection of SDS–PAGE gels of *O*-GlcNAcylated protein preparations from liver homogenates revealed an overall increase in *O*-GlcNAcylated proteins in diabetic rats (Fig. 3(A), right). The examination of protein immunoblot profiles contained in

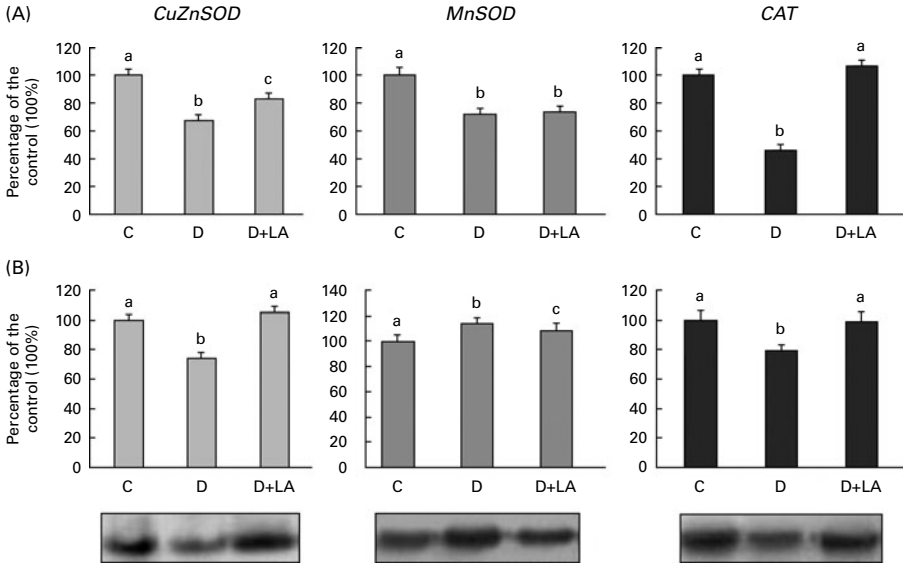

Fig. 2. Liver expression of CuZn superoxide dismutase (CuZnSOD), Mn superoxide dismutase (MnSOD) and catalase (CAT). (A) mRNA levels of *CuZnSOD*, *MnSOD* and *CAT*; (B) protein levels of CuZnSOD and CAT in the cytosol and that of MnSOD in the mitochondria. C, control rats; D, diabetic rats; D+LA, diabetic rats treated with α-lipoic acid (LA). Values are means from three experiments performed in triplicate, with their standard errors represented by vertical bars. Data were analysed by one-way ANOVA and statistical significance was tested by Duncan's test. [a,b,c] Mean values with unlike letters were significantly different ($P<0·0001$).

total liver homogenates (Fig. 3(B), left) with protein fractions obtained after WGA chromatography (Fig. 3(B), right) showed a very prominent increase in *O*-GlcNAcylated CAT species and a comparatively less-pronounced relative increase in *O*-GlcNAcylated CuZnSOD species (Fig. 3(C)). While liver homogenates from the diabetic rats contained a greater amount of MnSOD protein than that from the control rats (Fig. 3(B), left), they displayed a relatively small amount of *O*-GlcNAcylated MnSOD species (Fig. 3(C)), indicating that this protein underwent post-translational *O*-GlcNAcylation to a considerably lower extent than CuZnSOD and, in particular, CAT. LA administration promoted a general lowering of protein *O*-GlcNAcylation (Fig. 3(A), right), resulting in a reduction of *O*-GlcNAcylated CAT and CuZnSOD species

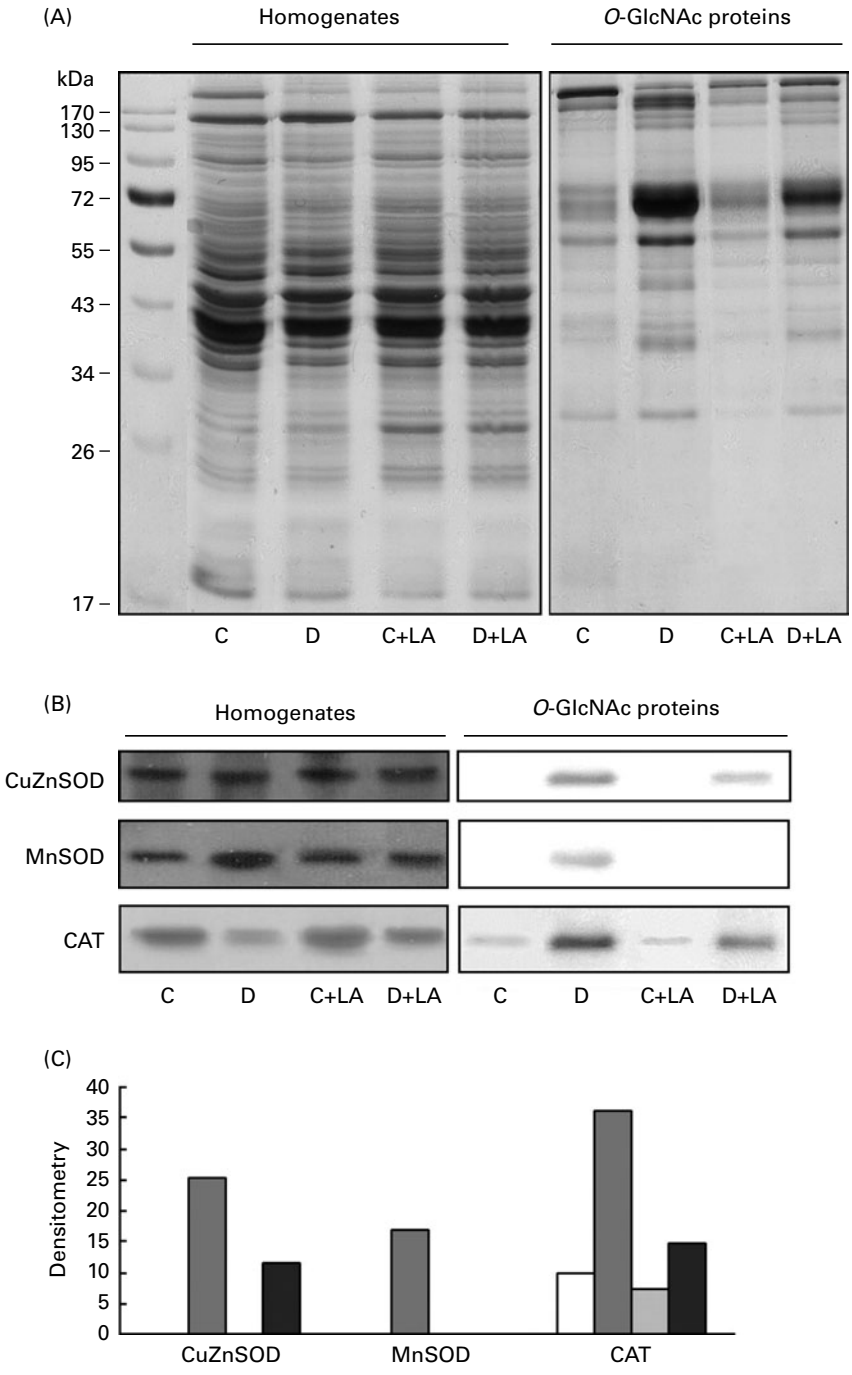

Fig. 3. *O*-Linked *N*-acetylglucosamine (*O*-GlcNAc) modification of liver proteins. (A) Electrophoretic profile of liver homogenates before and after elution from the WGA column. (B) Western blot analysis of liver homogenates and glycosylated proteins with anti-copper zinc superoxide dismutase (CuZnSOD), -manganese superoxide dismutase (MnSOD) and -catalase (CAT) antibodies. Representative gels and blots from three independent experiments are shown. (C) Densitometric analysis of immunoreactive bands detected by Western blot analysis of liver glycosylated proteins. C (□), control rats; D (▨), diabetic rats; C+LA (▩), control rats treated with α-lipoic acid (LA); D+LA (■), diabetic rats treated with LA.

and the complete absence of *O*-GlcNAcylated MnSOD enzyme (Fig. 3(B), right; Fig. 3(C)). LA treatment of control animals did not cause *O*-GlcNAcylation of the examined proteins.

Effect of α-lipoic acid treatment on factors involved in the regulation of antioxidative enzyme transcription

The metabolic and gene regulatory responses of the liver to inflammation, oxidative stress and diabetes are linked to the changes in activities of the transcription factors C/EBPβ and NF-κB and its subsequent effect on the expression of their target genes, including *CAT*, *CuZnSOD* and *MnSOD*. To study further the protective effect of LA administration on liver oxidative stress, we examined its effect on dynamic changes in C/EBP-β and NF-κB activation. The p65 subunit of NF-κB plays an essential role in inducing target genes for the NF-κB pathway in response to cell stress, with activating stimuli causing it to shuttle from the cytoplasm to the nucleus. While p65 was more abundant in the liver cytosol fraction of control rats, it was increased in the nuclei of diabetic rat liver (Fig. 4(A)). Likewise, C/EBPβ, which was present in 35, 30 and 35 kDa isoforms[34], was increased in the nuclei of diabetic rats. The observed induction of NF-κB and C/EBPβ in diabetic rats was accompanied by increased post-translational *O*-GlcNAcylation of these proteins (Fig. 4(B)). The LA treatment of diabetic animals promoted a partial inhibition of the nuclear translocation of p65, a decrease in nuclear C/EBPβ (Fig. 4(A)) and a reduced or completely abolished glycosylation of p65 and C/EBPβ, respectively. The transcription factors NF-κB and C/EBPβ can be activated by complex kinase pathways centred around p38 mitogen-activated protein kinase and ERK. Since *O*-GlcNAcylation of signalling kinases could modulate their ability to phosphorylate target proteins, we further verified whether p38 and ERK kinases are post-translationally modified by *O*-GlcNAcylation. While immunoblot analysis of total liver homogenates did not reveal quantitative changes in either p38 or ERK in any of the experimental groups (Fig. 4(B)), *O*-GlcNAcylated p38 and ERK species were observed in diabetic rats. In LA-treated diabetic rats, p38 and ERK *O*-GlcNAcylation reactions were significantly reduced. The LA treatment of control animals did not alter the *O*-GlcNAcylation of either p38 or ERK.

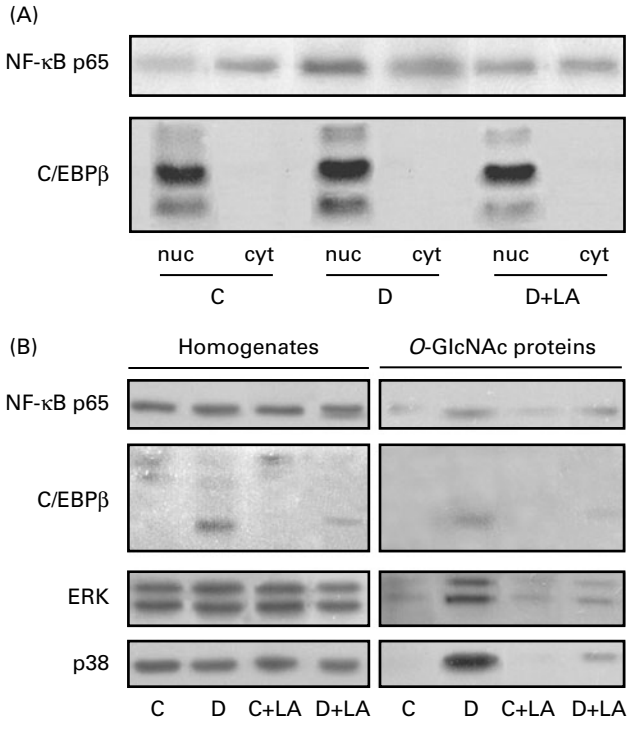

Fig. 4. Protein expression and *O*-linked *N*-acetylglucosamine (*O*-GlcNAc) modification of NF-κB protein 65 (p65), CCAAT/enhancer-binding protein β (C/EBPβ), extracellular signal-regulated kinase (ERK) and protein 38 (p38) in the liver of control, diabetic and α-lipoic acid (LA)-treated rats. (A) Western blot analysis on nuclear (nuc) and cytosol (cyt) proteins with anti-NF-κB p65 and -C/EBPβ antibodies. (B) Western blot analysis of liver homogenates and their glycosylated proteins with anti-NF-κB p65, -C/EBPβ, -ERK and -p38 antibodies. Representative blots from three independent experiments are shown. C, control rats; D, diabetic rats; C+LA, control rats treated with LA; D+LA, diabetic rats treated with LA.

Discussion

Increasing the knowledge of processes involved in oxidant–antioxidant homeostasis and the regulation of gene expression by the cell's redox state is expected to refine approaches to therapeutic interventions aimed at oxidative stress management[35]. In the present study, we provide evidence that a daily LA treatment of diabetic rats induces a trend towards the normalisation of CAT and SOD expression and function to physiological levels. LA administration reduces hepatic oxidative stress by recovering CuZnSOD and CAT enzymatic activities through gene up-regulation, and by lowering the levels of post-translationally *O*-GlcNAcylated CuZnSOD, MnSOD and CAT species. Examination of the potential mechanistic regulatory points revealed that LA administration to the diabetic rats decreased NF-κB p65 translocation and C/EBPβ levels, and at the same time reduced post-translational *O*-GlcNAc modifications of p65 and C/EBPβ, as well as of their upstream regulators, ERK and p38. These findings stress the importance of *O*-linked glycosylation in glucose toxicity that is exerted via its modulatory effects on cell signalling and transcriptional processes[36].

The liver plays a central role in the control of glucose homeostasis. The liver is subjected to regulation by insulin that maintains normal liver function. The loss of insulin signalling in hepatocytes leads to progressive hepatic dysfunction[37,38], resulting in inflammation, morphological and histopathological changes, necrosis and fibrosis[5], and ultimately causing acute liver failure[6]. The observed increased levels of liver enzymes, ALT and AST, at 4 weeks after STZ administration described herein revealed hepatocellular damage and changes in liver function, with the increase in the liver weight:body weight ratio, suggesting the onset of liver hypertrophy[39]. Liver hypertrophy relative to the body weight could be attributed to increased TAG accumulation leading to an enlarged liver due to an increased influx of fatty acids into the liver in hypoinsulinaemia, and the low capacity of excretion of lipoprotein from the liver due to deficient apoB synthesis. LA administration had a positive effect on ALT and AST levels in the diabetic liver as

a result of its normoglycaemic activity[18] and antioxidative mechanisms, as observed by decreased lipid peroxidation, normalised GSH:GSSG ratio and increased concentrations of protein SH groups. As a result, LA administration to the diabetic rats compensated for diabetes-induced hepatic cell damage, as observed by a significant decrease in liver cell DNA damage. The present results indicate that the mechanism through which LA administration exerted its antioxidant effect included an increase in the cell's endogenous defensive response, as observed by significantly elevated CuZnSOD and CAT enzymatic activities. Different models of diabetes have produced conflicting data regarding increases or decreases in antioxidant enzymes. Also, during STZ-induced diabetes, considerable differences in changes in the activities of antioxidant enzymes have been reported. While hepatic SOD activity is suppressed by the third or fourth weeks of diabetes, and is either normal or elevated by the eighth week after STZ treatment[1], CuZnSOD activity has been reported to decrease in the heart, liver and kidney[40,41]. Sadi *et al.*[42] obtained the results similar to those in the present study for CuZnSOD and CAT, while MnSOD activity was not changed in the diabetic liver[21]. Such discrepancies could result from different experimental conditions, including the age of the animals, diabetes duration or the type of the STZ-induced diabetic model. There is a general consensus that the experimental model of multiple low-dose, STZ-induced diabetes resembles more closely the *in vivo* state of insulinaemia, reflecting its autoimmune nature and resulting in diabetes onset, than the experimental model of diabetes induction with a single, high, cytotoxic dose of STZ[43]. Herein we present evidence that a rise in CuZnSOD and CAT enzymatic activities was due to increased amounts of both proteins as a consequence of the LA-promoted up-regulation of *CAT* and *CuZnSOD* gene transcriptional activities that were down-regulated in the STZ-induced diabetic rat liver when compared with the control. These findings suggest that the expression of hepatic CuZnSOD and CAT, while not excluding post-translational effects, was determined at the transcriptional level of control. A different effect of diabetic oxidative stress and the importance of an additional level of control were observed when MnSOD expression was examined. Whereas *MnSOD* gene transcription was down-regulated in diabetes, its protein level was increased. However, despite this relative increase, the specific enzymatic activity of MnSOD was lower in the diabetic liver compared with the control. While LA administration to the diabetic rats did not promote an observable change in *MnSOD* gene transcriptional activity, it resulted in a statistically significant decrease in MnSOD protein that was accompanied by a statistically significant elevation of MnSOD specific enzymatic activity. Taken together, these findings suggest that post-transcriptional and post-translational mechanisms play very important roles in determining MnSOD expression in response to changes in the cell's redox state, i.e. in diabetic and LA-treated diabetic rats. It is interesting to speculate that LA administration elicited a more direct and immediate response in CAT and CuZnSOD expression than in MnSOD expression which responded to improved cell metabolism due to the hypoglycaemic activity of LA.

At present, little is known about the regulation of SOD and CAT activity, particularly in the context of post-translational modifications. In the present study, we report for the first time that CuZnSOD, MnSOD and CAT are subjected to *O*-GlcNAc modification in diabetes. Post-translational *O*-GlcNAcylation is a dynamic process. It can disturb the balance between *O*-GlcNAcylation and *O*-phosphorylation, negatively affecting the functioning of target proteins[11,36,44]. As CAT activity is regulated by tyrosine phosphorylation in response to H_2O_2[45–47], it is possible that in diabetic conditions, *O*-GlcNAcylation lowered CAT activity by suppressing its tyrosine phosphorylation. Similarly, by modulating the local protein structure, *O*-GlcNAcylation of CuZnSOD and MnSOD could influence the effects of other post-translational modifications, such as phosphorylation which has been detected on SOD proteins[48], and/or it could partially or completely block their catalytic domains. Aside from the effects affecting enzymatic activity, the present results indicate that *O*-GlcNAcylation increased MnSOD stability. It has been suggested that glycosylation can contribute to protein stability[36], and while there is no obvious consensus sequence for *O*-GlcNAc binding, about 50 % of the known *O*-GlcNAc sites have Pro-Glu-Ser-Thr sequences that are associated with rapid degradation, suggesting that *O*-GlcNAcylation at these sites might slow or prevent degradation[49].

The activation of antioxidant enzyme genes is linked to the activities of complex kinase pathways which include protein kinase p38 and ERK that are involved in NF-κB[13,50] and C/EBPβ activation[14,51]. The transcription factor NF-κB, which is stimulated by ROS[52] and cytokines[53], assumes a key role in the liver, adipose tissue and central nervous system in the development of inflammation-associated metabolic diseases[54], and is responsible for induced CuZnSOD[55] and MnSOD expression[56]. The transcription factor C/EBPβ is implicated in basal CAT[57] and CuZnSOD transcription[58–60], with different C/EBPβ isoforms performing distinct functions in MnSOD transcription[61]. We found that STZ-induced diabetes was associated with enhanced nuclear translocation of NF-κB p65 and its increased *O*-GlcNAc modification in the rat liver. The finding that *O*-GlcNAc modification at Thr352 in response to high glucose concentrations inhibits the interaction of NF-κB with IκB[62] points to an explanation for the increased translocation of p65 observed in the diabetic liver. Consequently, in LA-treated diabetic rats, the decrease in p65 *O*-GlcNAcylation could have been responsible for its reduced nuclear translocation. The increased C/EBPβ protein level in the diabetic liver was suggested to be caused by the absence of insulin signalling[63] and disturbed glucose homeostasis[64]. *O*-GlcNAc modification of the *trans*-active 35 kDa C/EBPβ isoform was shown to interfere with its phosphorylation[14], and it can be speculated that the prominent *O*-GlcNAcylation of C/EBPβ in the diabetic liver affected its transcriptional activity. As observed with NF-κB, LA administration to the diabetic rats resulted in a lower C/EBPβ level and its decreased *O*-GlcNAcylation. These results, together with the finding that LA administration lowered post-translational *O*-GlcNAcylation of ERK and p38 in diabetic rats, point to the importance of this modification

in fine-tuning the functions of kinases and transcription factors involved in the regulation of antioxidant enzyme genes.

The present study focused on the examination of the mechanisms responsible for the beneficial effects of LA on the liver of STZ-induced diabetic rats treated with one daily dose (10 mg/kg) of LA for 4 weeks. However, the beneficial effects of LA on diabetic liver need further confirmation by performing experiments with several LA doses and at different time points of diabetes duration (6, 8, 12 and 16 weeks), as well as administering LA orally to rats instead of intraperitoneally and correlating the doses of LA doses between rats and humans. Also, it is essential to investigate the possible protective effects of LA on STZ-induced diabetic liver injury (by treating rats with LA before diabetes induction). On the basis of numerous clinical trials evaluating the efficacy of LA in treating diabetes-induced neuropathy, researchers have concluded that a daily oral dose of 600 mg LA provides an optimum risk: benefit ratio[20,65]. However, usage of appropriate pharmacological doses of LA for the treatment of diabetic patients is critical as some deleterious effects of LA cannot be excluded. In one case study reported by Ridruejo *et al.*[66], the clinical course and laboratory data point to cholestatic hepatitis after LA treatment (600 mg/d) of symptomatic diabetic neuropathy. This is the first report of liver injury induced by a dose of 600 mg/d of LA, which corresponds to the dose of 10 mg/kg used on rats in the present experiments. Ridruejo *et al.*[66] suggested the monitoring of liver enzyme levels during LA treatment every 1–3 months for the first 6 months, and then every 3–6 months. Although 600 mg/d is recommended as the safe LA dose for diabetes treatment in humans, some harmful effects are possible and precautions regarding safety and optimal LA doses must be undertaken.

In conclusion, hyperglycaemia in uncontrolled diabetic rat liver is associated with impaired antioxidant mechanisms and hepatocellular damage. Daily LA administration to diabetic rats reduced the negative effects of hyperglycaemia through hypoglycaemic activity and an antioxidative effect, which induced a trend towards the establishment of physiological levels of CAT and SOD expression. The pronounced increase in CAT and CuZnSOD enzymatic activities observed in LA-treated diabetic rats was due to the relief of enzyme inhibition caused by increased post-translational *O*-GlcNAcylation, and by transcriptional up-regulation, which is also supported by the decreased *O*-GlcNAcylation of upstream kinases and transcription factors. The comparatively, more subtle alterations in MnSOD expression in response to changes in the cell's redox environment were influenced by post-transcriptional and post-translational mechanisms. Given that antioxidant enzyme expression and function are dysregulated in diabetes, it was suggested that pharmacological modulation of key enzymes that are responsible for reducing the oxygen radical load is a potentially more effective approach than the use of systemic antioxidants[67]. Hence, the outcome of daily LA administration on antioxidant enzyme expression and function described herein warrants further investigation into its effects on the upstream end-points regulating antioxidant enzyme activities in the liver and other tissues.

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