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Lipoic acid improves glucose utilisation and prevents protein glycation and AGE formation

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The present study investigates the antiglycating effect of α-lipoic acid (LA) in high fructose-fed rats *in vivo* and its potential to inhibit the process of glycation *in vitro*. In addition, the effect of LA on glucose utilisation in rat diaphragm was also studied. Rats fed a high fructose diet (60% total calories) were administered with 35 mg/kg b.w, lipoic acid (LA) intraperitoneally for 20 days. The effects of LA on plasma glucose, fructosamine, protein glycation and glycated haemoglobin in high fructose rats and on *in vitro* glycation were studied. *In vitro* utilization of glucose was carried out in normal rat diaphragm in the presence and absence of insulin in which LA was used as an additive. The contents of glucose, glycated protein, glycated haemoglobin and fructosamine were significantly lowered on LA administration to high fructose-fed rats. LA prevented *in vitro* glycation and the accumulation of advanced glycation end products. Further LA enhanced glucose utilization in the rat diaphragm. This effect was additive to that of insulin and did not interfere with the action of insulin. The findings provide evidence for the therapeutic utility of lipoic acid in diabetes and its complications.

1. Introduction

Non-enzymatic glycation (NEG) of proteins has gained importance in recent years. The pathological effects of NEG are reflected in degenerative changes during ageing, chronic complications of diabetes and renal failure (Misur and Turk 2001). NEG of proteins are considerably faster and more intensive in diabetes mellitus, due to the persistently high concentrations of glucose. Glucose, the reducing sugar reacts with amines and basic amino groups of proteins non-enzymatically to form chemically unstable Schiff bases, which rearrange to form Amadori products (AP) (Turk 1997). The levels of these early glycation products change in response to blood glucose and are reflected in the analysis of glycated serum albumin and glycated hemoglobin (HbA_1C) which serve as important indicators of glycemic control. Amadori products degrade through rearrangement and autooxidation into α-dicarbonyl compounds forming inter- and intramolecular crosslinks of proteins known as advanced glycation end products (AGEs) (Bucala 1999). The irreversibly formed AGEs do not return to normal even when hyperglycemia is corrected and continue to accumulate over the lifetime of the protein. AGEs have been identified not only in long lived proteins but also in short lived proteins, including lipoproteins, plasma proteins and intracellular proteins (Vlassara et al. 1994). Accelerated cumulative modification of proteins and other biomolecules contribute to diabetic complications.

Pharmacologic agents that specifically inhibit the process of non-enzymatic glycation have been mostly investigated in terms of delaying the development and progression of diabetes-related complications. Efforts are on to identify a therapeutic tool to inhibit protein glycation. Amino guanidine (Edelstein and Brownlee 1992), aspirin, acetaminophen and ibuprofen have been shown to decrease glycation of lens proteins and prevent diabetic cataract in rats (Blakytny and Harding 1992).

α-Lipoic acid (LA), a naturally occurring antioxidant, is a co-factor of the α-ketoacid dehydrogenase complexes in the mitochondria. LA is reduced *in vivo* to its dithiol form dihydrolipoic acid (DHLA), which also possesses biological activity. LA-DHLA pair functions as an unique and effective system in recycling other antioxidants namely vitamins C and E and elevating glutathione levels (Evans and Goldfine 2000). In addition, LA has effects on glucose metabolism. LA improves glucose metabolism and insulin sensitivity in type 2 diabetic patients (Jacob et al. 1995). LA enhances glucose transport into the skeletal muscle isolated from Zucker rats (Hernriksen et al. 1997). In clinical trials LA plays a beneficial role in preventing certain complications such as cataract and neuropathy (Borenshtein et al. 2001; Ford et al. 2001).

The above data prompted us to investigate the possible inhibitory effect of LA on glucose-induced protein glycation and AGE formation *in vitro* using bovine serum albumin (BSA) as the model protein and *in vivo* in high fructose-fed rats. Further, the effect of LA on glucose utilization in control rat diaphragm *in vitro* was investigated and compared with that of insulin.

2. Investigations and results

Table 1 gives the levels of plasma glucose, glycated haemoglobin, glycated protein and fructosamine in control

Table 1: Effect of lipoic acid supplementation on plasma glucose, fructosamine and glycated haemoglobin in control and experimental animals

	CON	FRU	FRU + LA	CON + LA
Glucose (mM)	4.73 ± 0.35	5.40 ± 0.18*	4.88 ± 0.36#	4.78 ± 0.39
Fructosamine (mM)	0.84 ± 0.06	1.36 ± 0.28*	0.93 ± 0.06#	0.86 ± 0.02
Glycated protein (μM)	2.26 ± 0.21	3.44 ± 0.25*	2.37 ± 0.36#	2.27 ± 0.15
Glycated haemoglobin (% total Hb)	0.24 ± 0.07	0.63 ± 0.09*	0.31 ± 0.03#	0.25 ± 0.04

Values are mean ±SD of 6 rats from each group; * – Compared with CON; # – Compared with FRU; Significant at $P < 0.05$ (ANOVA)

Table 2: Effect of LA on glucose utilization in rat hemidiaphragm incubated with glucose *in vitro*

Groups	LA concentration	Insulin concentration	Glucose utilization (μM/g/h)
CON	–	–	15.40 ± 0.62
LA_{50}	50 nM	–	23.03 ± 0.65*
LA_{100}	100 nM	–	27.65 ± 1.23*
LA_{50} + INS	50 nM	0.2 U/ml	42.50 ± 4.04#
LA_{100} + INS	100 nM	0.2 U/ml	48.51 ± 0.78#
INS	–	0.2 U/ml	32.73 ± 1.88

The incubation medium contained 0.04 M sodium phosphate (pH 7.2), 0.005 M potassium chloride, 0.004 M magnesium chloride, 0.006 M glucose, 0.08 M sodium chloride
Values are mean ±SD of 6 experiments
* – Compared with CON; # – Compared with INS; Significant at $P < 0.05$ (Students t-test)

and fructose-fed animals. The levels were significantly elevated in fructose-fed rats, while LA treated fructose rats showed near normal values. In rats fed control diet, administration of LA did not produce significant alterations as compared to control group.

Table 2 shows the effect of LA on *in vitro* glucose utilization by rat diaphragm. LA at various concentrations (50 nM and 100 nM) caused a significant increase in glucose utilization as compared to control. The experiments were performed by incubating hemidiaphragms with LA in the presence or absence of insulin (0.2 U/ml). It was seen that the insulin effect was larger than that of LA. However, in the presence of both LA and insulin the utilization of glucose was greater than that with insulin alone. Hence the effects of insulin and LA on glucose utilization by the diaphragm are additive.

The inhibitory effect of LA on non-enzymatic glycation of albumin was assessed upon addition of LA to the reaction mixtures in concentrations of 25, 50 and 100 nM (Fig. 1). The glycation of albumin was assumed to be 100% in the absence of LA. A 54.35% inhibitory effect was observed for a LA concentration of 25 nM, while at a concentration of 50 nM LA showed a stronger (71.41%) inhibitory effect. At 100 nM, LA produced the strongest inhibitory effect on glycation, reducing it by 86.2%. Accordingly the inhibitory effect of LA was found to depend on the concentration used.

Fluorescence of samples after 15 days and 30 days of incubation are shown in Fig. 2. LA at a concentration of 100 nM was found to inhibit maximally. After 15 days of incubation the sample containing 100 nM LA showed a significant reduction in fluorescence as compared to other

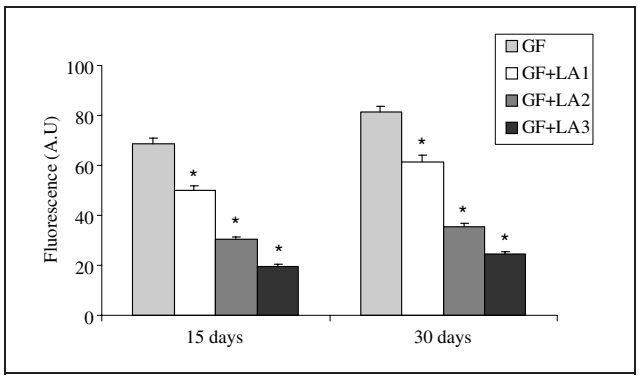

Fig. 2: Fluorescence of the samples after 15 days and 30 days incubation. Reaction mixtures contained glucose (25 mM, G), fructose (25 mM, F) and LA (25, 50 and 100 nM, LA1, LA2, LA3). Fluorescence was recorded at 450 nm upon excitation at 350 nm. Values are means ±SD of six experiments; * – significant at $P < 0.05$ when compared to GF (Students t-test).

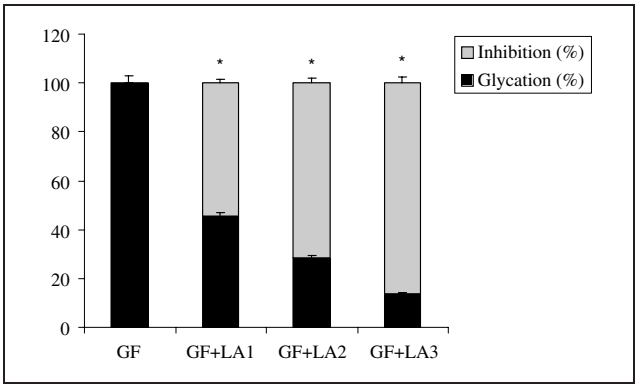

Fig. 1: The inhibitory effect of LA on non-enzymatic glycation of albumin after 30 days incubation. Reaction mixtures contained glucose (25 mM, G), fructose (25 mM, F) and LA (25, 50 and 100 nM, LA1, LA2, LA3).
GF-glucose + fructose; GF + LA1-glucose, fructose + LA (25 nM), GF + LA2-glucose, fructose + LA (50 nM); GF + LA3-glucose, fructose + LA (100 nM); Values are means ±SD of six experiments; * – significant at $P < 0.05$ when compared to GF (Students t-test)

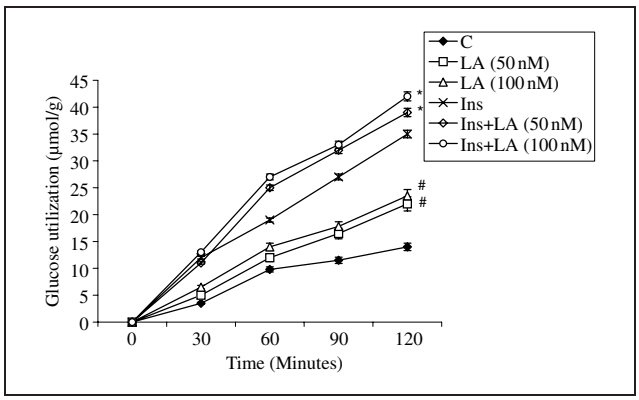

Fig. 3: Effect of LA on glucose utilization by the diaphragm in the presence and absence of insulin with time. Both LA and insulin individually increased glucose utilization. When present together the effect was greater.
CON – Control, LA – α-lipoic acid, INS – Insulin; Values are means ±SD of six experiments; * – significant at $P < 0.05$ when compared with CON (Students t-test); # – significant at $P < 0.05$ when compared with CON (Students t-test)

concentrations of LA. After 30 days of incubation, the reaction was characterized by intensive formation of non-enzymatic glycation end products, the following results were recorded: LA inhibited 54% of the fluorescent product formation at the concentration of 25 nM, 69% at 50 nM and 81% at the concentration of 100 nM of LA.

Fig. 3 depicts the glucose uptake by the diaphragm with time. Under basal conditions the rate of glucose utilization fell off with time. Insulin caused an immediate increase in glucose disappearance and the insulin effect was present during the entire 2 h of incubation. The effect of LA was found to be completely different. LA had no action on glucose uptake during the first hour but exerted a stimulatory effect during the subsequent period of incubation. Hence LA was capable of maintaining the ability of the tissue to metabolize glucose but, unlike insulin, it does not immediately increase glucose utilization. In the presence of both LA and insulin the utilization of glucose was greater than when they were present alone.

3. Discussion

The major findings of the present study are the prevention of glycation both *in vitro* and *in vivo* and enhancement of glucose utilisation *in vitro* by LA. Chronic hyperglycemia in diabetes enhances the production of reactive oxygen species (ROS) from glucose autoxidation and from oxidative fragmentation of Amadori products and deoxyosones (Wolff et al. 1991). ROS such as superoxide, hydrogen peroxide and hydroxyl radical are formed during protein glycation. Glycated proteins produce nearly 50 times more free radicals than non-glycated proteins and are likely to oxidize key cellular constituents like lipids (Mullarkey et al. 1990). Glucose autoxidation forms dicarbonyl products, which mediate glyoxidation, site-specific protein damage and lipoxidation. In the process more ROS are generated establishing further vicious and interactive cycles of molecular damage.

LA has been considered as an antioxidant by its ability to inhibit oxidative processes. LA is an unique antioxidant, which potentially can react directly with reactive oxygen species even in its oxidized form (Coleman et al. 2001). This differential property, which the other endogenous antioxidants such as GSH, vitamins C and E do not possess, makes LA a more powerful antioxidant than any other endogenous compound. LA is known to scavenge ROS, chelate metal ions, regenerate endogenous antioxidants GSH, vitamins C and E and repair oxidized proteins (Packer et al. 1995). Thus, the antioxidant function of LA could contribute to the inhibition of protein glycation and subsequent AGE formation. Previously Jain and Lim (2000) reported that LA inhibits peroxidation and protein glycation in RBC exposed to high concentrations of glucose.

LA enhanced the utilisation of glucose in the rat diaphragm. The effect was greater when both LA and insulin were present than when they were present individually. Studies have shown the stimulatory effects of LA on glucose transport and oxidation. For instance, administration of LA has been shown to rise basal and insulin-stimulated glucose-uptake in skeletal muscle of glucose intolerant non-insulin dependant diabetic animals (Jacob et al. 1996; Henriksen et al. 1997) and in cardiac muscle of streptozotocin diabetic rats (Strodter et al. 1995). As a co-factor for the mitochondrial α-ketoacid dehydrogenases, lipoic acid can potentially influence the rate of glucose oxidation. Studies show that LA can increase glucose uptake by increasing the translocation which apparently is mediated via increased kinase activity of the insulin receptor, insulin receptors substrate-1, phosphatidyl inositol-3 kinase and protein kinase B (Yaworsky et al. 2000). This suggests that LA can influence early signaling pathways of insulin action to promote glucose utilisation.

High dietary fructose can increase the formation of glyceraldehydes and metholglyoxal (Thornalley 1993), malondialdehyde and 4-hydroxynonenal, the products of lipid peroxidation (Dargel 1992). The carbonyl carbon of these aldehydes reacts readily with the free amino and sulfhydryl groups of functional enzymes and membrane proteins forming Schiff bases or amino compounds and alters their function (Schauenstein et al. 1977). Studies show that LA can lower tissue aldehydes either by stimulating glucose metabolism thereby decreasing the formation of reactive aldehydes or by increasing the concentrations of sulfhydryl substances like cysteine or glutathione that can bind to aldehydes (Vasdev et al. 2000). Further LA may itself have the capacity to bind to aldehydes owing to the presence of the −SH group.

The effects of LA on protein glycation and glucose utilization could be related to each other. The decrease in the availability of glucose may cause a decrease in ROS production through reductions in glucose autooxidation and formation of lipoxygenase products or respiratory activity and hence protein glycation.

The concentrations of LA used in the study are based on the physiological concentrations of LA, which is about 5–25 nmol/g in mammalian tissues (Alvarez and Boveris 1995). Normal concentrations of LA could be important in preventing diabetic pathology. Significant reductions in the levels of LA were observed in rat liver during alloxan-induced experimental diabetes (Natraj et al. 1984). Deficiency of LA in type 2 diabetic patients (Maxwell et al. 1997) and in patients with diabetic retinopathy (Harnett et al. 2000) are also observed.

The present findings show that LA improves glucose utilisation and reduces glycation of proteins, both of which are critical in the management of diabetes and thus provide a mechanism for LA action in diabetes. It is worthy to note that LA is a drug prescribed for diabetic complications in some countries like Germany (Ziegler et al. 1999).

4. Experimental

4.1. Chemicals

Lipoic acid was obtained from the Sigma chemical company, St Louis, MO, USA and bovine serum albumin (BSA) was obtained from Merck Pvt Ltd, Mumbai, India. All other chemicals were of analytical grade and were from Himedia laboratories Pvt. Ltd, Mumbai, India.

4.2. Incubation

The procedure for *in vitro* glycation of BSA was adopted from Vinson and Howard (1996) with modifications. The incubation mixtures in a final volume of 2 ml contained 14 mg BSA, 25 mM fructose, 25 mM glucose and either Tween 80 as control or the inhibitor, lipoic acid at concentrations 25 nM, 50 nM or 100 nM in screw-capped test tubes. BSA, fructose and glucose solutions were dissolved in 0.1 M Tris buffer (pH 7) containing 0.02% sodium azide. Lipoic acid was freshly prepared by dissolving it in Tween-80. The mixtures were incubated in quadruplicates in a water bath at 37 °C for 3 to 30 days.

4.3. Measurement of early glycation products

The mixtures were subjected to extensive dialysis in the cold to remove sugars from the solution. Glycated proteins were measured by the method of Rao and Pattabiraman (1990). The proteins were precipitated with trichloroacetic acid and hydrolysed with oxalic acid. The product was reacted with phenol and the colour formed was measured using glucose as the standard.

4.4. Measurement of AGEs by AGEs linked specific fluorescence

The fluorescence intensity of the sample was measured at 450 nm after excitation at 350 nm (Yanagisawa et al. 1998) using a fluorescence spectrophotometer. Fluorescence was expressed as the relative fluorescence intensity in arbitrary units (A.U.). An unincubated blank containing BSA, sugars and LA was used as the blank.

4.5. Animal study

Male Wistar rats of body weight ranging from 170–190 g were obtained from the Central Animal House, RMMCH, Annamalai University. They were housed two per cage under controlled conditions on a 12 h light and dark cycle. The animals used in the present study were cared for as per the principles and guidelines of the Ethical Committee of Animal Care of Annamalai University in accordance with the Indian National Law on animal care and use (Reg. No. 166/1999/CPCSEA) and all procedures were cleared by the committee.

4.6. Supplementation study

The antiglycating effect of LA was tested in the high fructose-fed animal model. The animals were divided into the following groups. Control animals (CON) received the commercial diet and tap water *ad libitum*. Fructose-fed animals (FRU) received a fructose-enriched diet and tap water *ad libitum*. Fructose fed animals (FRU + LA) received the fructose diet and LA (35 mg/kg b.w/day dissolved in saline) intraperitoneally. Control animals (CON + LA) received the commercial diet and LA (35 mg/kg b.w/day dissolved in saline) intraperitoneally. The animals were maintained in their respective groups for 20 days. The animals were killed by decapitation and blood was collected. Glycated haemoglobin in whole blood and protein glycation in plasma was measured by the method of Rao and Pattabiraman (1990). Glucose (Sasaki et al. 1972), and fructosamine (Johnson et al. 1983) were also analyzed in plasma.

4.7. In vitro glucose utilization

In vitro utilization of glucose in rat diaphragm was determined according to the method of Haugaard and Haugaard (1970). Hemidiaphragms were removed from the control rats and collected in a cold solution containing 0.04 M sodium phosphate (pH 7.2), 0.005 M KCl, 0.004 M $MgCl_2$ and 0.083 M NaCl. The pieces of diaphragm were blotted in a filter paper and weighed. The tissues were incubated in a medium containing 0.04 M sodium phosphate (pH 7.2), 0.005 M KCl, 0.004 M $MgCl_2$, 0.006 M glucose and 0.08 M NaCl at 37 °C for 24 h. Glucose utilization under basal conditions was determined in control samples without LA and insulin. The concentration of LA when present was either 25 nM or 50 nM and that of insulin was 0.2 U/ml. The degree of glucose utilized during the 24 h *in vitro* incubation was determined by measuring the glucose levels in the medium at 0 h and 24 h using O-toluidine method.

4.8. Statistical analysis

The values are expressed as means ±SD. All the grouped data were statistically evaluated by one way analysis of variance (ANOVA) or student's t-test. The significance was set at $P < 0.05$.

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