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Lipoic acid and diabetes: Effect of dihydrolipoic acid administration in diabetic rats and rabbits

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Abstract. Relative α-lipoic acid content of diabetic livers was considerably less than that of normal livers as determined by gas chromatography. It was not possible to detect any dihydrolipoic acid in the livers. Biochemical abnormalities such as hyperglycaemia, ketonemia, reduction in liver glycogen and impaired incorporation of [2-^{14}C] -acetate into fatty acids in alloxan diabetic rats were brought to near normal levels by the oral or intraperitoneal administration of dihydrolipoic acid. The effect of α-lipoic acid was comparable to that of dihydrolipoic acid in reducing the blood sugar levels of diabetic rabbits during a glucose tolerance test.

The results suggest that the mode of action of lipoic acid was through stimulation of pyruvate dehydrogenase.

Keywords. α-Lipoic acid; dihydrolipoic acid; gas chromatography; alloxan diabetic rats; blood glucose; acetoacetate; liver glycogen; fat synthesis.

Introduction

α-Lipoic acid is a vital cofactor in the multienzyme complexes that catalyze the oxidative decarboxylation of α-keto acids (e.g., pyruvate, α-ketoglutarate and branched chain α-keto acids). There is an impairment of pyruvate oxidation in diabetes as evidenced by the decreased levels of the active form of pyruvate dehydrogenase (EC 2.4.1.1) (Kerbey *et al.,* 1976). Consequently, a large proportion of the acetyl coenzyme A (CoA) needed for energy generation is obtained through fatty acid oxidation resulting in an increased ratio of acetyl CoA/CoA in the mitochondria and increased production of ketone bodies (Antko, 1972; Kerbey *et al.,* 1976; Randle *et al.,* 1977). Further, the increased gluconeo-genesis in diabetes has been shown to be due to enhanced activities of several cytosolic enzymes such as phosphoenolpyruvate carboxykinase (EC 4.1.1.32), fructose 1,6-bisphosphatase (EC 3.1.3.11) and the mitochondrial enzyme pyruvate carboxylase (EC 6.4.1.1) (Prinz and Seubert, 1964; Wilmhurst and Manchester, 1970). In streptozotocin induced diabetes, pyruvate carboxylase activity is significantly increased whereas a larger proportion of pyruvate dehydrogenase is in its inactive state (Weinberg and Utter, 1980). Thus, it becomes apparent that the metabolism of pyruvate is a crucial regulatory step in diabetes because it is a metabolite located at the crossroads of the three major pathways impaired in diabetes, *i.e*., glycolysis, gluconeogenesis and

Abbreviations used: DTO, Dihydrolipoic acid; LS_2, lipoic acid; CoA, coenzyme A; NMR, nuclear magnetic resonance.

amino acid biosynthesis. Further, Wada *et al.* (1960) have reported that the levels of α-lipoic acid in the blood and liver in alloxan diabetic rats are considerably reduced. In view of this, it was considered useful to evaluate the role of α-lipoic acid in diabetes.

α-Lipoic acid has been estimated by several procedures including microbiological methods (Wada *et al.,* 1960; Herbert and Guest, 1975), gas chromatographic (Shih and Steinsberger, 1981; White, 1981) and high performance liquid chromatography (Howard and McCormick, 1981). The microbiological assay does not distinguish between α-lipoic and dihydrolipoic acid and further the assay does not work in the presence of excess glucose. Hence, this procedure can be expected to give erroneous results in diabetes because of the rather high glucose concentrations. In the present study we have estimated the levels of α-lipoic and dihydrolipoic acid in the livers of normal and alloxan diabetic rats using a gas chromatographic procedure. The effect of α-lipoic and dihydrolipoic acid administration on some of the major biochemical abnormalities in diabetic rats has also been investigated.

In this context, it may be mentioned that several investigators have attempted to reduce blood sugar in human diabetes by administration of α-lipoic acid (Pagliaro, 1956; Pagliaro and Furitano, 1956; Greco, 1957; Brusa and Serafini, 1958; Zueva, 1970). No efforts have been made to elucidate the possible mode of action of lipoic acid. In the present study, it was found that α-lipoic acid was considerably reduced in the liver of diabetic rats and administration of α-lipoic and dihydrolipoic acid had a beneficial effect on all the biochemical aberrations measured in diabetic rats.

Materials and methods

DL α-lipoic acid was purchased from British Drug House Ltd., Poole, England. Alloxan monohydrate was obtained from SD Fine Chemicals, Bombay. [2-^{14}C]-Acetate was procured from Bhabha Atomic Research Centre, Bombay, and all other reagents were of analytical grade. Male rats and rabbits used in this study were an inbred Haffkine strain.

Analytical methods

Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker WH 90 instrument operating under ASPECT 2000 control at 90 and 23 MHz for ^{1}H and ^{13}C respectively using $CDC1_3$ as solvent.

For gas chromatographic analysis, methylation was carried out by reacting lipoic acid (106mg, 0·5mmol) or dihydrolipoic acid (106mg, 0·5mmol) with anhydrous methanolic HCl (5%, 2ml) at 60°C for 20 min. After reaction, the mixture was flushed with N_2 gas to dryness. The methyl ester was redissolved in $CHC1_3$ for analysis.

Methylated compounds were analysed on a Hewlett Packard 5730 A gas Chromatograph. The column was 3% SP-1000 coated on Supelcoport (100–120). N_2 was the carrier gas and the compounds were detected by flame ionization. The column temperature was programmed from 150°-240°C at a rate of 2°C/min. The temperature of the detector and injector were 300°C and 250°C respectively.

Radioactivity measurements were performed on a Packard Tricarb liquid scintillation spectrometer. Blood and urinary sugar were determined according to the method of Somogyi (1952).

Preparation and estimation of dihydrolipoic acid

The procedure used by Hager and Gunsalus (1953) was modified as follows: DL α-lipoic acid (212 mg, 1 mmol) was dissolved in 0·25 M potassium bicarbonate (12 ml). Sodium borohydride (48 mg, 1·5 mmol) was added to the stirred solution keeping the temperature below 5°C. The reaction mixture was acidified to pH 2·0 with chilled 5 N HCl and extracted with benzene. The organic layer was washed with water, dried over anhydrous sodium sulphate and the solvent distilled under reduced pressure.

The sulphydryl content of dihydrolipoic acid was estimated using the procedure of Alexander (1958).

Isolation of lipoic acid from rat liver

Rats of two age groups (25 and 45 days old) were used in this study. Some rats in each age group were made diabetic by subcutaneous injection of alloxan (180 mg/kg) in acetate buffer (0·1 M, pH 5·4). Rats excreting more than 10 g/1 sugar in urine were used in the study.

α-Lipoic acid was isolated from the livers of rats as described by Carreau (1979). Normal and diabetic rats (4 each in the two age groups) were sacrificed, the livers removed, washed with saline, blotted dry and weighed. Wet liver (1g) was homogenized in Potter-Elvehjem homogenizer with 7 ml of water at 4°C. Homogenization was continued first with the addition of 17 ml methanol followed by 17 ml chloroform and finally 8 ml of water. The resulting mixture was centrifuged at 2000*g* at 0°C for 20 min. Three layers separated: (i) upper aqueous methanolic layer, (ii) interphase and (iii) lower chloroform layer. The layers were separated, the interphase was homogenized with a 2:1 mixture of water and chloroform and centrifuged as before. The aqueous and chloroform layers were separated and mixed with the corresponding layers from the first centrifugation. This operation was repeated four more times.

The pooled aqueous methanolic extracts were concentrated on a rotary evaporator and the residue was dissolved in 0·5 ml of 6 N HCl and hydrolyzed in a sealed tube at 110°C for 16 h. After evaporating the HCl, the residue was extracted with chloroform. The chloroform layer was evaporated with a stream of N_2 gas and the lipoic acids were separated on thin layer chromatography with chloroform: methanol:water:formic acid (75:25:4:1;v/v). Pure lipoic and dihydrolipoic acid (visualised on a separate plate with a solution of 0·5g of palladium chloride, 100ml water, 400ml ethanol and 10 drops of cone. HCl) regions were cut out, extracted with chloroform: methanol (1:1, v/v). The extract was filtered, the solvent evaporated and the residue esterified with methanolic HCl.

The methylated compounds were filtered through a microcolumn (0·5×3cm) of silicic acid (100 mesh) and the samples used for gas chromatography.

Glucose tolerance test in normal and diabetic rabbits

Four normal and six alloxan diabetic rabbits weighing about 2·5kg were starved for 18h and their fasting blood sugar determined. Dihydrolipoic acid or α-lipoic acid (l00μmol/kg) was injected intraperitoneally as a suspension in 0·5ml of 0·8% saline in two normal and three alloxan diabetic rabbits. The remaining animals received 0·5ml of saline alone. Two h later a 40% solution of glucose in water (2g/kg) was administered orally. Blood sugar (ear vein) was estimated at hourly intervals upto 4 h.

Blood sugar in diabetic rats

Nine alloxan diabetic rats weighing about 100g were divided into two groups. Dihydrolipoic acid (500 μmol/kg) was administered orally as a suspension in saline in six animals and three animals receiving saline alone served as controls. Blood was drawn by cardiac puncture at 0,2 and 4h following dihydrolipoic acid administration and the sugar content determined.

Acetoacetate levels in diabetic rats

Dihydrolipoic acid (500μmol/kg) was administered as a suspension in saline to four alloxan diabetic rats. Blood was collected by cardiac puncture before and 24 h after dihydrolipoic acid administration and acetoacetate was estimated by the method of Walker (1954).

Liver glycogen in normal and diabetic rats

Six normal and 6 alloxan diabetic rats weighing about 100g were distributed into two groups. Dihydrolipoic acid (500μmol/kg) as a suspension in saline was injected intraperitoneally to three normal and three diabetic rats. The remaining animals were injected saline alone. The animals were sacrificed 24h later, the livers removed, washed with a solution of saline, blotted dry and weighed. The glycogen content was determined according to the method of Hassid and Abraham (1956).

[2-^{14}C] -*Acetate incorporation into liver lipids in normal and diabetic rats*

Dihydrolipoic acid (500μmol/kg) was injected intraperitoneally into three normal and four diabetic rats. Three normal and 4 diabetic rats administered saline only served as controls. Two h later [2-^{14}C]-acetate (4μCi/100) g) was injected in all the animals. Twenty four h after dihydrolipoic acid administration, the animals were sacrified, the livers removed, washed with a solution of saline, blotted dry and weighed. The wet livers were homogenized in chloroform: methanol (2:1 v/v) in an Omnimixer for 5 min at 3000 rpm. The organic layer was separated by filtration, washed with a 0·03% magnesium chloride solution and then with water. The solvent was distilled under reduced pressure and the residue (lipids) was weighed to a constant weight. The total radioactivity in the residue was determined.

Results

GC analysis of methyl lipoate and methyl dihydrolipoate

Methyl lipoate gave a single peak with a retention time of 32·9 min (figure 1, panel A). However, methyl dihydrolipoate gave two peaks with retention times of 28·4 and 32·9 min (figure 1, panel B). The relative intensities of these two peaks were 40:56. The latter corresponds to methyl lipoate and the former presumably to methyl dihydrolipoate. It was therefore necessary to establish whether the methyl dihydrolipoate was contaminated with methyl lipoate or whether during GC analysis it was getting oxidized to methyl lipoate. Purity analysis was carried out using ^{13}C NMR. From this analysis it was concluded that methyl dihydrolipoate was 95% pure. Therefore, it appears that during GC analysis 56% of the methyl dihydrolipoate gets oxidized. As long as the extent of this reaction is taken into account, GC analysis of liver samples can be used for quantitative estimation of dihydrolipoic acid levels.

Levels of α-lipoic and dihydrolipoic acid in rat livers

The presence of α-lipoic acid was detected in all the liver samples tested by this method. Despite the presence of unidentified peaks in the gas chromatogram, α-lipoic acid could be identified as a distinct peak with a retention time of 32 min (figure 1, panel C). Inclusion of a known quantity of methyl lipoate as an external standard showed that the intensity of the peak with a retention time of 32 min was increased by the corresponding amount (figure 1, panel D). The relative peak areas before and after addition of standard methyl lipoate was a measure of the lipoic acid content in the sample (table 1). In alloxan diabetic rat livers the α-lipoic acid content was one fourth of that found in normal livers.

Dihydrolipoic acid could not, however, be detected in any of the samples analysed. Taking into account the sensitivity of this method, it was estimated that the levels of dihydrolipoic acid, if present, must be less than 0·5 μg/g tissue.

The results of the glucose tolerance test are shown in table 2. Dihydrolipoic acid and

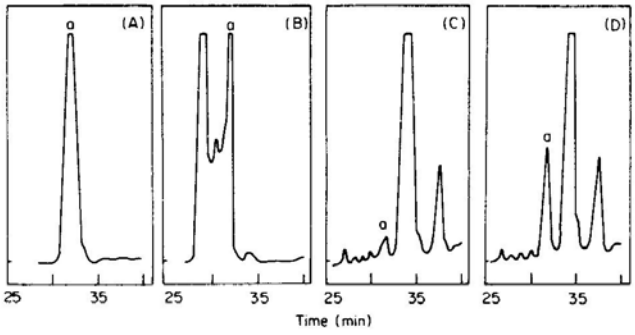

Figure 1. Gas chromatography analysis of methyl lipoate and methyl dihydrolipoate. Panel A, Pure methyl lipoate (a). Panel B, Methyl dihydrolipoate. Panel C, Liver extract. Panel D, Same extract with externally added methyl lipoate.

Table 1. α-Lipoic acid content of livers from normal and alloxan diabetic rats.

Age (days)	α-Lipoic acid content (μg/g wet tissue)		
	Normal (N)	Diabetic (D)	Ratio (N/D)
25	13·1 ± 0·05	3·85 ± 1·6	3·4
45	8·0 ± 2·1	2·05 ± 0·6	3·9

All values are means of 4 rats ± S.E

Table 2. Glucose tolerance test in normal and diabetic rabbits.

	Blood glucose mg/100 ml (mean ± SE) Hours				
	0	1	2	3	4
Normal (2)	72	152	105	87	90
Normal (2) + DTO	74	129	102	80	84
Diabetic (3)	257 ± 34	449 ± 62	494 ± 17	485 ± 37	446 ± 46
Diabetic (3) + DTO	245 ± 35	375 ± 34	319 ± 67	287 ± 12	276 ± 49
P value			< 0·1	< 0·05	< 0·05
Diabetic (3)	145 ± 15	261 ± 28	271 ± 27	248 ± 24	188 ± 6
Diabetic (3) + LS_2	152 ± 12	179 ± 15	212 ± 15	178 ± 15	162 ± 22
P value		< 0·05		< 0·05	

Numbers in parenthesis indicate the number of animals.

α-lipoic acid administration in alloxan diabetic rabbits brought about a significant reduction in the blood sugar levels in a paired '*t*' test at all the time points tested. Furthermore, the profile of the glucose tolerance test resembled the normal profile more closely. Neither dihydrolipoic acid nor α-lipoic acid had any significant effect on blood glucose levels in normal rabbits.

Similarly, it was found that dihydrolipoic acid administered orally also brings about a significant reduction in the blood sugar levels of diabetic rats (table 3). The mean reduction in the blood sugar levels at the end of 2 and 4h was 22% and 38% respectively.

Acetoacetate levels in the blood were reduced by a greater than two fold margin following dihydrolipoic acid administration in diabetic rats (table 4).

In addition to bringing about a reduction in the blood sugar and acetoacetate levels in diabetic rats, dihydrolipoic acid increases the liver glycogen (table 5) and [2-^{14}C]-acetate incorporation into liver lipids (table 6) by a greater than two fold margin. At the

Table 3. Blood glucose levels in diabetic rats.

	Blood glucose mg/100 ml (mean ± SE) Hours		
	0	2	4
Diabetic (3)	345 ± 51	343 ± 40*	333 ± 22*
Diabetic + DTO (6)	421 ± 55	328 ± 51**	261 ± 38***

Numbers in parenthesis indicate the number of animals.
* Not significantly different from 0 h reading.
** Significantly different from 0 h reading at $P < 0{\cdot}1$.
*** Significantly different from 0 h reading at $P < 0{\cdot}01$.

Table 4. Acetoacetate levels in blood of diabetic rats.

	Acetoacetate (mg/100 ml)	*P* value
Normal	0·78 ± 0·1	
Diabetic	11·5 ± 1·0	< 0·001
Diabetic	11·5 ± 1·0	< 0·01
Diabetic + DTO	4·1 ± 1·2	

All values are means of 4 animals ± SE.

Table 5. Liver glycogen in normal and diabetic rats.

	Liver glycogen g per cent	*P* value
Normal	3·29 ± 0·35	
Normal + DTO	2·89 ± 0·21	NS*
Diabetic	0·74 ± 0·06	
Diabetic + DTO	1·69 ± 0·27	< 0·01

All values are means of 3 animals ± S E.
* Not significant.

same time, these two constituents are not affected significantly in normal rats administered dihydrolipoic acid.

Discussion

It is evident that α-lipoic acid levels are significantly reduced in rat livers during alloxan diabetes. Wada *et al.* (1960) had noted a similar reduction in α-lipoic acid in diabetic

Table 6. Incorporation of [2–^{14}C]-acetate into liver lipids.

	CPM incorporated × 10^3		
	Per g wet tissue	Per g of fat	*P* value
Normal (3)	5·9 ± 1·3	141·6 ± 21	
Normal + DTO (3)	4·5 ± 0·4	109·6 ± 16	NS*
Diabetic (4)	1·5 ± 0·3	43·5 ± 9·1	
Diabetic + DTO (4)	3·7 ± 0·5	85·5 ± 5·3	< 0·05

Numbers in parenthesis indicate the number of animals ± SE.
* Not significance.

rats. However, they had employed the microbiological assay which has several limitations as pointed out earlier. We have also shown that dihydrolipoic acid could not be detected in the livers of normal or alloxan diabetic rats. It is interesting to note that Shih and Steinsberger (1981) employing a similar extraction followed by GC analysis also failed to detect any dihydrolipoic acid in chick liver. It appears reasonable to conclude therefore, that α-lipoic acid exists mainly as the disulphide and not the sulphydryl form in liver.

Lipoic acid exists predominantly in the protein bound form as the ε-aminolysyl amide (Reed, 1966). The method of extraction employed in this study is designed to measure only protein bound lipoic acid. It is, of course, possible that some of the α-lipoic acid may be degraded during acid hydrolysis. However, it is expected that this loss does not affect the ratio of α-lipoic acid levels in normal and alloxan diabetic rat livers.

Results of α-lipoic and dihydrolipoic acid administration show that the four major biochemical abnormalities in diabetes *viz.,* hyperglycaemia, ketonemia, reduced glycogen and fat synthesis, have been corrected to a significant extent by the administration of dihydrolipoic acid in diabetic rats. On the other hand, dihydrolipoic acid did not affect any of these parameters to a significant extent in normal animals. In addition, dihydrolipoic acid increases the tolerance to glucose in diabetic rabbits during a glucose tolerance test. In this system the effect of α-lipoic acid is very similar to that of dihydrolipoic acid.

Diabetes is a disease characterised by an aberration of glucose metabolism and transport (Foster, 1978; Karnielli *et al.,* 1981). Insulin administration has a general beneficial effect on both these parameters. It is difficult to assess the relative contributions made by these two effects in diabetes because of the difficulty in studying them in isolation. However, it is safe to say that both effects are extremely important for the proper management of glucose levels in the blood. The mechanism of action of insulin in improving glucose metabolism has been ascribed generally to the stimulation of several enzymes involved in glycolysis, tricarboxylic acid cycle and fatty acid biosynthesis (Denton *et al.,* 1981). The pivotal enzyme at the crossroads of these three pathways is pyruvate dehydrogenase (EC 1.2.4.1) which is a multienzyme complex catalyzing the oxidative decarboxylation of pyruvate to acetyl CoA. In view of the

importance of this reaction in the metabolism of glucose, the role of insulin in regulating the activity of this enzyme is of considerable interest.

The predominant mode of regulation of pyruvate dehydrogenase has been shown to be by a phosphorylation (inactive enzyme) and dephosphorylation (active enzyme) mechanism. Insulin brings about a reversible dephosphorylation, thus converting the inactive to the active form (Hughes *et al.,* 1980). The importance of the activation of pyruvate dehydrogenase in mediating the hypoglycaemic effect of insulin is evidenced by the fact that dichloroacetate, an inhibitor of pyruvate dehydrogenase kinase also brings about an activation of pyruvate dehydrogenase, and has been shown to induce hypoglycaemia in diabetic and starved normal rats (Whitehouse and Randle, 1973; Blackshear *et al.,* 1974). Thus, one may presume that atleast a part of the hypoglycaemic effect of insulin is mediated by the activation of pyruvate dehydrogenase.

The question, therefore, is whether the beneficial effects of dihydrolipoic acid and α-lipoic acid administration in diabetes is mediated through insulin secretion or directly through the activation of pyruvate dehydrogenase. Since alloxan administration results in a near total destruction of *β*-cells of the pancreas, neither α-lipoic or dihydrolipoic acid release any extra insulin over and above that released by the high glucose levels present in diabetic rats (Malaisse *et* al., 1982).

Evidence for the alternative hypothesis stems from the fact that the activity of pyruvate dehydrogenase is lowered in alloxan diabetes (Kerbey *et a*l., 1976). Administration of insulin restores only a part of the activity of this enzyme (Stansbie *et al.,*1980; Wieland *et al.,* 1971). This points to an irreversible diminution of a part of this enzyme activity in alloxan diabetic rats, perhaps due to lower levels of α-lipoic acid in the liver. It would be reasonable to conclude therefore, that administration of α-lipoic acid or dihydrolipoic acid restores the activity of this enzyme thus alleviating some of the biochemical aberrations in diabetes. Definitive evidence indicating the enhancing effect of lipoic acid on liver pyruvate dehydrogenase Bill be presented in a subsequent communication.

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