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Failure of short-term γ-linolenic acid treatment to reduce urinary calcium loss of diabetic rats

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Abstract Calcium re-absorption in the kidney is impaired in streptozotocin (STZ) diabetic rats, thereby causing hypercalciuria. Increased calcium loss starts within 1–2 days after induction of diabetes and reaches a plateau after 2 weeks. The excessive calcium excretion was previously shown to be reduced by treatment with γ-linolenic acid (GLA) or evening primrose oil rich in GLA. However, in these studies, the animals were pretreated for several weeks before injection of STZ. In the present study we investigated whether GLA can reduce calcium excretion when treatment starts at the same time as induction of diabetes. Rats were made diabetic with 60 mg/kg STZ and at the same time food was fortified with 0.4% GLA for the treatment group. A control group was treated with vehicle alone and given standard feed only. Urine was collected from animals in metabolism cages every 3rd day for a period of 26 days. The diabetic group increased their food and water consumption, and urine and faeces production as compared to the control group. The urinary loss of Ca, Mg, Zn, Na, K and creatinine was markedly increased in the diabetic group as compared to the control. GLA treatment, however, did not affect any of these variables. Analysis of fatty acids in kidneys of the rats showed an increased concentration of GLA in the treated group as compared to the two non-treated groups. We conclude that GLA treatment must commence before STZ injection in order to attenuate diabetes-induced hypercalciuria.

Key words Hypercalciuria · Diabetes mellitus · γ-linolenic acid

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Introduction

In diabetes mellitus, a set of secondary abnormalities in various organs have been described. These include not only classical diabetic nephropathy, retinopathy, neuropathy and cardiopathy but also a specific bone disease [14]. A decrease in bone mineral content has been noted in both humans [5] and rats with diabetes [13]. In early human and experimental diabetes mellitus, hypercalciuria is a common characteristic and contributes to the alterations in calcium homeostasis in the disease [1, 7, 11, 15]. The detailed mechanism for hypercalciuria in diabetes is not known, but evidence suggests that the lesion is in the thick ascending limb of Henle or the distal segment of the renal tubule [3, 6].

The use of γ-linolenic acid (GLA) has been suggested for prevention of diabetes-induced complications [2]. This is thought to circumvent the reduced activity of delta-6-desaturase found in diabetes [10]. This enzyme converts linoleic acid to GLA which is then further metabolised to arachidonic acid (AA). By normalising the lipid profile with GLA, effects may take place via altered membrane fluidity or signaling systems such as prostaglandin production.

GLA, in the form of evening primrose oil and purified oil, has been shown to prevent diabetes-induced hypercalciuria in rats made diabetic by streptozotocin [4, 9, 12]. In all of these studies the animals were pretreated over several weeks before induction of diabetes. In studies of diabetic neuropathy, nerve conductance speed could be increased with only 2 weeks of GLA-supplementation, commencing 6 weeks after induction of diabetes [2]. This suggests that a short GLA treatment period is sufficient for treating some of the complications of diabetes. The aim of the present study was to test the hypothesis that GLA would be effective in preventing hypercalciuria when administrated to rats at the same time that diabetes was induced with streptozotocin.

Materials and methods

Animals

Experiments were performed using female Sprague-Dawley rats (Charles River Laboratories, Wilmington, Kent, UK) which were maintained under a constant 12-h light/dark photoperiod (lights on 0800 hours) and a temperature of 21–23 °C. They had free access to food (see below) and water throughout the study. All work was performed in accordance with the UK Animals (Scientific Procedures) Act 1986.

Rats were bought at 5 weeks of age. At 8 weeks, they were placed in glass metabolism cages (Metabowls, Jencons Scientific Ltd, Hemel Hemstead, Herts, UK) and were allowed an acclimatisation period of 7 days before the first collection of urine. While the rats were in metabolism cages they were provided with deionised water. A record of water and food consumption and production of urine and faeces was made over 24 hours every 3rd day for 26 days.

Animal diets

Rats were either fed normal CRM pellets (Special Diet Services, Whitham, Essex, UK) or CRM pellets supplemented with 0.4% GLA (Scotia Pharmaceuticals Ltd, Guilford, Surrey, UK). The latter diet was prepared by dissolving GLA in diethyl ether and soaking pellets in the solution. The ether was allowed to evaporate in a fume-cupboard overnight. GLA-enriched food was freshly prepared every second day.

Experimental design

Diabetes was induced in 10 rats 1 day after the first urine collection by a single intraperitoneal injection of streptozotocin (STZ; Sigma-Aldrich Co. Ltd, 60 mg/kg dissolved in citrate buffer). Three rats were injected with vehicle alone and were fed normal CRM diet throughout the experiment (control group). Diabetes was confirmed by the presence of glucosuria (> 5.5 mmol/l; Labstix, Ames, Slough, Berks, UK) 2 days after injection and later in the experiment by blood sampling from the tail vein. GLA treatment started in 5 of the 10 diabetic rats immediately after STZ injection and continued throughout the experiment (GLA group). The remaining five diabetic rats received CRM diet alone (diabetic group).

At the end of the experiment, the rats were killed by cervical dislocation and blood was collected from the descending aorta. Kidneys was dissected, weighed, frozen in liquid nitrogen and stored at −80 °C for later analysis of GLA and arachidonic acid (AA). This was kindly performed by Scotia Pharmaceuticals (Nova Scotia, Canada).

Analysis

Urinary samples were analysed for sodium and potassium by flame photometry (Corning EE1 model 450; Scientific and Medical Products Ltd, Manchester, UK), and for calcium, magnesium and zinc by atomic absorption spectrophotometry (Perkin-Elmer 3100; Beaconsfield, Bucks, UK). Creatinine was analysed by the Jaffe-reaction using an autoanalyser (Monarch Autoanalyser, Instrumentation Laboratories).

Statistical analysis

Data are presented as mean ± SEM. Statistical analysis of the data was performed using the SPSS PC software package.

Results

Injection of STZ resulted in all 10 animals becoming diabetic as confirmed by the presence of glucose in the urine 2 days after injection, and by hyperglycemia in blood measured at 18 days (control 8 ± 0.4; diabetic 43 ± 2; GLA 46 ± 2 mmol/l glucose) and in serum at 26 days after injection (control 7.6 ± 0.2; diabetic 40 ± 2; GLA 33 ± 7 mmol/l glucose). There were no significant differences between the body weights of the rats in the three groups at the start or end of the experiment (Table 1).

Water consumption and urine production were increased within 2 days after STZ injection in the diabetic

Table 1 Data collected from rats in metabolism cages at 1 day before (−1) and 26 days after streptozotocin injection. The three groups were comparable for all variables before streptozotocin injection. No differences between diabetic and GLA groups were found at any point of the experiment. $^{**}P < 0.01$ vs control, $^{***}P < 0.001$ vs control

	Day	Control ($n = 3$) Mean ± SEM	Diabetic ($n = 5$) Mean ± SEM	Diabetic + GLA ($n = 5$) Mean ± SEM
Body weight (g)	−1	222 ± 12	223 ± 5	234 ± 7
	26	288 ± 15	268 ± 14	256 ± 17
Water consumption (ml/24 h)	−1	33 ± 3	30 ± 3	30 ± 5
	26	38 ± 10	307 ± 17***	364 ± 20***
Food consumption (g/24 h)	−1	21 ± 0.5	23 ± 4	22 ± 1
	26	23 ± 4	57 ± 7**	63 ± 4
Urine output (ml/24 h)	−1	22 ± 1	16 ± 2	17 ± 4
	26	23 ± 6	293 ± 24***	347 ± 42***
Faeces output (g/24 h)	−1	9 ± 1	11 ± 1	12 ± 1
	26	11 ± 1	29 ± 5**	31 ± 3***
Urinary Mg output (mmol/24 h)	−1	0.26 ± 0.04	0.26 ± 0.02	0.2 ± 0.05
	26	0.24 ± 0.05	0.96 ± 0.1**	0.89 ± 0.14**
Urinary Zn output (mmol/24 h)	−1	0.16 ± 0.04	0.16 ± 0.02	0.14 ± 0.02
	26	0.12 ± 0.02	2.3 ± 0.2***	1.8 ± 0.3**
Urinary Na output (mmol/24 h)	−1	2.3 ± 0.04	2.4 ± 0.05	2.1 ± 0.2
	26	1.9 ± 0.4	6.0 ± 0.5***	6.0 ± 0.5***
Urinary K output (mmol/24 h)	−1	3.8 ± 0.06	3.7 ± 0.1	3.6 ± 0.3
	26	3.3 ± 0.07	9.7 ± 0.7***	10.3 ± 0.5***
Urinary creatinine output (μmol/24 h)	−1	61 ± 0.6	61 ± 2	54 ± 5
	26	86 ± 8	192 ± 8***	191 ± 8***

and GLA groups in comparison to the control group, and continued to rise throughout the experiment. Food consumption and faeces production were also increased in both STZ injected groups. GLA supplementation did not affect any of these variables (Table 1).

The calcium concentration in urine decreased in the diabetic and GLA groups (data not shown). However, as the urine volume increased, there was an increase in urinary calcium loss in the two diabetic groups as compared to the control group, reaching a 10-fold difference by the end of the experiment (Fig. 1). The calcium excretion began to rise as early as 2 days after STZ injection and was significant in both diabetic groups by day 5 as compared to the control group ($P < 0.05$, ANOVA). Urinary calcium losses were not different in the Diabetic group and the GLA group (Fig. 1). Similarly, the urinary losses of magnesium, zinc, sodium, potassium and creatinine were all increased in the diabetic group compared to the control group, but were not affected by GLA supplementation (Table 1). By the end of the experiment, kidney weights were increased in diabetic rats but GLA supplementation had no effect on this variable either (control 1.0 ± 0.1 g; diabetic 1.7 ± 0.1 g; GLA 1.7 ± 0.1 g).

In order to confirm that dietary GLA had been consumed and absorbed by the rats, GLA and AA were analysed in their kidneys. The percentage of GLA between control and diabetic groups did not differ per total lipids or any lipid fraction analysed (Table 2). The GLA group, however, had markedly increased GLA concentration in all lipid fractions. The concentration of AA in total lipids was not different between kidneys of the control and diabetic groups. However, AA was found to be decreased in free fatty acids and phospholipids but increased in triglycerides and cholesterol esters in the Diabetic group as compared to the control group. GLA supplementation of diabetic rats significantly increased AA in the free fatty acids and phospholipids, but not in triglycerides and cholesterol ester.

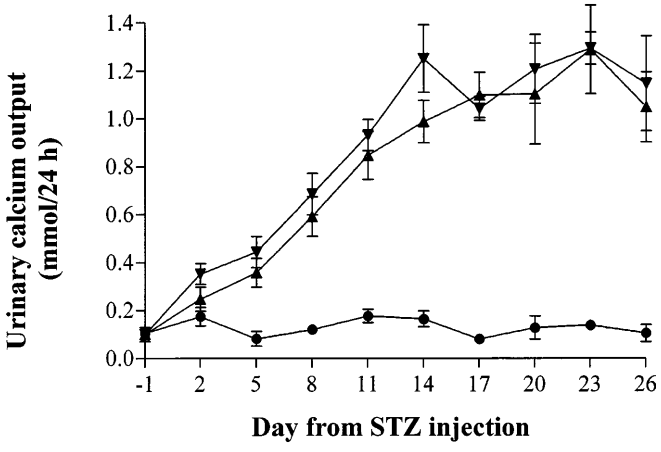

Fig. 1 Urinary calcium excretion in control (●), diabetic (▲) and γ-linolenic acid (GLA, ▼) groups. The diabetic and GLA groups were significantly increased compared to the control group from day 5 ($P < 0.05$) and onwards ($P < 0.001$). No differences between the diabetic and GLA groups were found at any time point

Discussion

The data presented in this study show that supplementation of diabetic rats with GLA does not decrease urinary calcium loss when treatment commences at the onset of diabetes. This is in sharp contrast to previous reports where rats had been pre-treated with evening primrose oil or pure GLA for 4 weeks or more before STZ injection [4, 9, 12]. Tulloch et al., for example, fed male Lewis rats a fat-deficient diet supplemented with 8.6% evening primrose oil (containing 9.2% GLA) for 4 weeks before making them diabetic with STZ. After 6 weeks of diabetes, calciuria was found to be reduced to normal levels in the supplemented group [12]. In a similar study with pregnant animals, Garland et al. [4] fed Sprague-Dawley rats 8.6% evening primrose oil for 5 weeks before making them diabetic with STZ and mating. The treatment reduced urinary calcium output to approximately half of that seen in untreated diabetic rats by the end of pregnancy [4]. Similarly, feeding Sprague-Dawley rats 0.5 g/kg per day of pure GLA for 5 weeks before administration of STZ and mating also reduced hypercalciuria when compared to untreated diabetic animals [9].

Table 2 Percentage of γ-linolenic acid (*GLA*) in total lipid and its fractions in kidneys after 26 days of streptozotocin injection, and percentage of arachidonic acid (*AA*) in total lipid and its fractions. No difference in GLA was found between Control and Diabetic groups, but GLA was significantly increased in all fractions in the GLA-treated diabetic group. AA in total lipid was not affected by diabetes or GLA-treatment, while that in the phospholipid and free fatty acid fractions was decreased, and that in the triglycerides and cholesterol ester was increased in the Diabetic group compared to Control. GLA treatment significantly increased AA in phospholipids and free fatty acids only. $*^1P < 0.05$ vs control; $*^2P < 0.01$ vs control; $*^3P < 0.001$ vs control; $*^4P < 0.05$ vs diabetic; $*^5P < 0.001$ vs diabetic

	n =	Total lipid	Total phospholipid	Free fatty acids	Triglycerides	Cholesterol ester
GLA						
Control	3	0.14 ± 0.04	0.12 ± 0.02	0.18 ± 0.04	0.14 ± 0.05	0.00 ± 0.00
Diabetic	5	0.14 ± 0.06	0.08 ± 0.00	0.29 ± 0.02	0.16 ± 0.16	0.00 ± 0.00
Diabetic + GLA	5	0.89 ± 0.06 $*^{3,}$ $*^5$	0.42 ± 0.02 $*^{3,}$ $*^5$	1.36 ± 0.10 $*^{3,}$ $*^5$	2.01 ± 0.25 $*^{3,}$ $*^5$	1.31 ± 0.53
AA						
Control	3	15 ± 0.9	28 ± 0.2	21 ± 0.6	0.5 ± 0.03	1.1 ± 0.58
Diabetic	5	17 ± 1.1	25 ± 0.4 $*^3$	14 ± 0.4 $*^3$	1.6 ± 0.2 $*^1$	6.9 ± 0.98
Diabetic + GLA	5	20 ± 1.1	26 ± 0.4 $*^{1,}$ $*^4$	16 ± 0.4 $*^{3,}$ $*^5$	2.5 ± 0.48 $*^2$	6.0 ± 2.2

The contrasting effect of GLA on calcium output in diabetes between current and previous studies could be due to two main factors. Firstly, the dose of GLA in this study was lower than that in the evening primrose studies. However, it was similar to our previous work with pure GLA which did decrease hypercalciuria in pregnant rats [9]. Moreover, the renal concentration of GLA and AA in our supplemented group at the end of the experiment was increased when compared to untreated diabetes and was similar to or even higher than that seen in the study of Pikgongarm et al. (unpublished observation). Secondly, the total treatment time in the current study was shorter than in any of the previous experiments and hence may have been too short for the expected action to take place. The most likely explanation for the present findings, therefore, is that it takes several days for GLA to achieve its protective effect, and that during that period of uncontrolled diabetes an irreversible alteration in renal calcium transport occurs. It is notable that insulin will only reverse the hypercalciuria of experimental diabetes mellitus if treatment is started shortly after induction of the disease [1, 8].

We conclude, based on previous findings and our current results, that GLA requires a pre-treatment period in order to reduce the hypercalciuria of diabetic rats. The cause is most likely to be due to a slow onset of the therapeutic effect of the GLA, which fails to overcome the rapidly induced (and permanent) lesion in renal calcium transport produced from the diabetes.

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