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Effect of *myo*-inositol supplementation on the development of renal pathological changes in the Cohen diabetic (type 2) rat

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Summary A lower concentration of intracellular *myo*-inositol has been implicated in the development of diabetic nephropathy. This was based on short-term studies showing that early administration of aldose reductase inhibitors or *myo*-inositol supplementation reduces increased glomerular filtration rate and partly reduces increased urinary albumin excretion in streptozotocin diabetic rats. We studied the effect of long-term (4 months) administration of 1 % *myo*-inositol supplement to the Cohen diabetic (type 2) rat on the development of nephropathy and renal Na^+-K^+-ATPase. This treatment reduced the increased renal Na^+-K^+-ATPase activity but had no effect on blood glucose levels, body weight, increased kidney weight, or creatinine clearance and did not prevent or reduce the development of renal glomerular pathology. There was no correlation between the level of Na^+-K^+-ATPase activity and the degree of nephropathy. It is possible that the renal pathological changes are due to metabolic and humoral factors resulting from hyperglycaemia, other than *myo*-inositol depletion. The fact that *myo*-inositol treatment had no effect on the development of renal pathological changes but was shown to have a beneficial effect on restoring impaired conduction velocity and on the disruption of structural elements in the nerve indicates that the effect of the biological changes ensuing from hyperglycaemia vary in different tissues depending on local conditions. [Diabetologia (1995) 38: 899–905]

Key words Non-insulin-dependent diabetes mellitus (type 2), *myo*-inositol, glomerulosclerosis, Na^+-K^+-ATPase, Cohen-diabetic rat.

It has been suggested that polyol pathway hyperactivity contributes to the pathogenesis of diabetic renal complications [1–4]. This was based on the observation that treatment with aldose reductase inhibitors and *myo*-inositol supplements reduces the increased glomerular filtration rate [5, 6] and partly reduces increased urinary albumin excretion [6–8] in diabetic animals.

Most of these observations have been made in short-term type 1 streptozotocin diabetic rats (SZT-D). The purpose of the present study was to evaluate the effect of long-term supplementation of *myo*-inositol in the Cohen diabetic rat (type 2) on the development of pathological changes and Na^+-K^+-ATPase activity in the kidneys.

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Abbreviations: Pi, Inorganic phosphate; IDDM, insulin-dependent diabetes mellitus; NIDDM, non-insulin-dependent diabetes mellitus; STZ-D, streptozotocin diabetic rats; GS, glomerulosclerosis; PG, prostaglandin.

Materials and methods

Animals. The Cohen diabetic rat shares essential features with human non-insulin-dependent diabetes such as genetic and environmental disposition [9], an early phase of hyperinsulinaemia, and insulin resistance, followed by hypoinsulinaemia [10], a decreased number and sensitivity of insulin receptors

Table 1. Clinical and biochemical characteristics of Cohen diabetic rats supplemented with *myo*-inositol (group I)

No	Pathology No	Body weight (g)	Blood glucose (mmol/l)	Creatinine clearance (ml · min^{-1} · 100 g body weight)	Proteinuria 24 h (mg)	(g) kidney 100 g/body weight	Glomerulosclerosis grade			Na^+-K^+-ATPase (μmol Pi · mg protein^{-1} · h^{-1})	
							N[c]	2+	3–4+	cortex	medulla
1	1327	312	20.7	0.333	327	0.641	–	–	4+		
2	1328	294	29.4	0.245	86	0.510	N	–	–		
3	1326	268	19.6	0.321	113	0.642	–	–	4+		
4	1350	360	23.6	0.166	37	0.417	N	–	–		
5	1349	400	18.4	0.293	11	0.533	N	–	–		
6	1352	360	23.6	0.175	14	0.416	–	2+	–		
7	1351	360	20.3	0.250	30	0.416	–	–	3+		
8	1314	360	19.2	0.580	49	0.841	–	–	3+		
9	1313	360	29.4	0.325	36	0.500	N	–	–		
10	1316	335	22.7	0.382	26	0.570	N	–	–	42	104
11	1317	310	23.2	0.290	12	0.484	N	–	–		
12	1318	291	19.7	0.249	41	0.484	–	–	3+		
13	1315	355	18.2	0.270	108	0.563	–	–	3+	47	49
14	1319	280	17.8	0.357	193	0.714	–	–	3+		
		322 ± 10	21.9 ± 0.97[a]	0.303 ± 0.026[a]	77.4 ± 22.7[a]	0.553 ± 0.032[a]	6	1	7[b]	89:2 = 45	153:2 = 77

[a] vs downward line $p < 0.05$
[b] vs diabetic non-supplemented $-\chi^2$ ($df = 2$) = 1.9957
[c] N = Normal (o change)

Table 2. Clinical and biochemical characteristics of Cohen diabetic rats (group II) and the downward, non-diabetic line (group III)

				Cohen diabetic rats (group II)							
No	Pathology No	Body weight (g)	Blood glucose (mmol/l)	Creatinine clearance (ml · min^{-1} · 100 g body weight)	Proteinuria 24 h (mg)	(g) kidney/ 100 g body weight	Glomerulosclerosis grade			Na^+-K^+-ATPase (μmol Pi · mg protein^{-1}/h^{-1})	
							N[c]	1+	3–4+	cortex	medulla
1	1344	372	20.1	0.266	22	0.484	N	–	–		
2	1345	294	20.6	0.058	23	0.558	N	–	–		
3	1343	370	30.2	0.272	28	0.354	N	–	–		
4	1329	300	23.2	0.267	157	0.550	–	–	3+		
5	1347	330	23.7	0.374	11	0.870	N	–	–		
6	1357	255	19.9	0.497	45	0.606	–	–	3+		
7	1330	210	20.3	–	91	0.870	–	–	4+		
8	1348	230	24	0.102	33	0.373	N	–	–		
9	1320	265	28.4	0.272	41	0.562	–	–	3+		
10	1321	365	19.7	0.250	75	0.556	–	–	3+	72	220
11	1323	312	21.2	0.234	35	0.536	–	–	3+		
12	1324	318	20.3	0.233	57	0.541	–	–	4+		
13	1322	291	29.3	0.220	344	0.550	–	–	4+	49	72
14	1325	268	21.2	0.334	146	0.641	5		4+		
		300 ± 13[a]	23 ± 0.953[a]	0.262 ± 0.0291[a]	79.1 ± 22.78[a]	0.575 ± 0.379	5		9[b]	121:2 = 61	292:2 = 146
				Downward, non-diabetic line group III							
1–6	1352–1357	381 ± 14.1[c,d]	6.71 ± 0.25[c,d]	0.92 ± 0.046[c,d]	11.9 ± 2.2[c,d]	0.347 ± 0.012[c,d]	N	–	–	49 77	127 131.7
										126:2 = 63	258.7:2 = 129

[a] vs downward line $p < 0.05$
[b] vs diabetic supplemented $-\chi^2$ ($df = 2$) = 1.9957
[c] vs diabetic non-supplemented $p < 0.05$; N = Normal (o change)
[d] vs diabetic supplemented $p < 0.05$

[11], a metabolic response to hypoglycaemic drugs [12], and renal and retinal complications [13]. The procedure that leads to the development of diabetes in this model has been described in detail elsewhere [9]. Briefly, two lines of this strain were selected from parental "Sabra" rats (Hebrew University albino strain) by feeding a copper-deficient (1.2 parts per million, analysed by atomic absorption, spectrophotometry), sucrose-rich diet (sucrose 72%, vitamin-free casein 18%, butter 4.5%, corn oil 0.5%, salt mixture USP No. II 5%, water- and fat-soluble vitamins). Offspring with abnormal glucose tolerance (upward selection) were brother-sister mated and developed hyperglycaemia, glycosuria and diabetic vascular complications. The downward-selected or so-called resistant line remained normal under the sucrose-rich diet. Diabetic animals

with spontaneous blood glucose levels over 16.6 mmol/l were used in this study.

Animal groups

Group I. (Table 1) Sixteen randomly-selected three-month-old diabetic animals continued to consume the high-sucrose diet which was supplemented with 1 % *myo*-inositol (Sigma, St. Louis, Mo., USA) for 4 months, up to the age of 7 months.

Group II. (Table 2) Fifteen 3-month-old diabetic animals were fed the high-sucrose diet up to the age of 7 months.

Group III. (Table 2) Six male normal non-diabetic animals of the downward line were fed the animal house stock chow diet up to the age of 7 months.

At the age of 7 months in all groups, the blood glucose, 24-h urinary protein excretion and creatinine clearance were determined. The animals were killed under deep pentobarbitol anesthaesia. The kidneys were removed and the left kidney was weighed, and the weight of the kidney per 100 g body weight was calculated. Specimens for histological studies were taken.

In six animals of groups I, II and III, kidney specimens were taken for evaluation of Na^+-K^+-ATPase activity. In groups 1 and II, the kidney specimens of animals numbers 9, 10, 11 and 12, showing low urinary protein excretion (12–75 mg/24 h) and of animals numbers 13 and 14, with high urinary protein excretion (108–344 mg/24 h) were pooled separately for determination of Na^+-K^+-ATPase activity.

Preparation of microsomes. Preparation of microsomal ATPase was carried out according to Jorgensen and Skou [14]. The pooled tissues were homogenized in 10 volumes of a medium containing 0.25 sucrose, and 2 mmol/l EDTA buffered with 5 mmol/l Tris-HCl to a pH of 7.4–7.5. The homogenate was centrifuged at 7,000 *g* for 15 min; the supernatant was decanted and the sediment centrifuged at 48,000 *g* for 40 min. The pellet was resuspended in an equal volume of the above solution and again homogenized in 10 volumes of desoxycholate 0.1 % containing 2 mmol/l EDTA and 25 mmol/l Tris-HCL (pH 7.0). After incubation at room temperature for 30 min, the suspension was centrifuged at 25,000 *g* for 30 min. The pellet was suspended in the above sucrose-EDTA-Tris. This final suspension was frozen at –20 °C overnight and assayed the next day.

Assay of ATPase. ATPase activity was determined by the amount of inorganic phosphate (Pi) released during incubation at 37 °C in a shaking, thermostatic bath, as previously described [15]. All assays were run in duplicates. The Pi release was studied with and without K^+ in the medium. The standard incubation medium consisted of: (in mmol/l) NaCl 100, KCl 10, $MgCl_2$ 4, ATP 4. Enzymatic activity was stopped by the addition of 10 % trichloracetic acid. Pi was determined according to the method of Fiske and Subbarow [16]. Enzymatic protein was assayed according to Lowry et al. [17]. Na^+-K^+-ATPase was estimated as the difference of Pi release with and without K^+ in the medium.

Creatinine clearance. Fluid intake and urine output were measured at 24-h intervals for 2 consecutive days in animals kept separately in metabolic cages. On the third day, blood was taken from the tail. Creatinine in the blood and urine was measured by an automatic picric acid method (Roche, Cobas Mira Diagnostica, Basel, Switzerland) and evaluated per 100 g body weight.

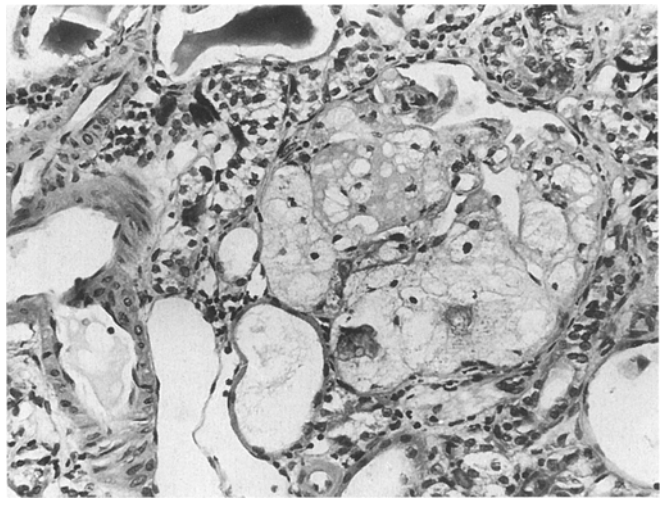

Fig. 1. Histologic section of kidney of diabetic animal with grade IV lesion showing xanthomatous lesions in a glomerulus

Blood glucose. Animals were fasted overnight; the next day food was served at 08.00, and 10.00 hours a blood sample was taken from the tip of the tail in heparinized test tubes. Blood glucose levels were determined by a SMAC II system (Technion Instrument Corp, New York, N. Y., USA).

Pathological evaluation and grading. Whole transverse sections of both kidneys were evaluated blind by the pathologist (E. R.), and approximately 300 glomeruli were screened in each case. The degree of glomerulosclerosis (GS) was graded according to the number of glomeruli involved (focal or generalized), and the extent of glomerular involvement (segmental or diffuse). The degree of histopathological changes was rated from 0 to 4: 0 = no change; grade 1 = focal, 15–30 % of glomeruli segmentally and/or diffusely involved; grade 2 = focal, 30–50 % of glomeruli segmentally and/or diffusely involved; grade 3 = generalized, 50–100 % of glomeruli segmentally and/or diffusely involved; grade 4 = generalized, with secondary tubular atrophy and/or dilatation and mild interstitial fibrosis, with xanthomatous (Fig. 1) and/or lipohyaline glomerular lesions. None of the affected kidneys were found to have Armanni-Ebstein lesions.

Statistical analysis

Results were expressed as mean ± SEM. The unpaired Student's *t*-test and the chi-square test were used. Differences were considered significant at $p < 0.05$.

Results

During the experimental period two animals from the treated diabetic group and one animal from the untreated group died in the cage.

Plasma glucose and body weight

Blood glucose levels ranged between 19.7 and 30.2 mmol/l, and were not affected by the *myo*-inositol supplement, 21.9 ± 0.97 mmol/l in the treated and 23 ± 0.95 mmol/l in the untreated diabetic group.

Body weight was also unaffected by *myo*-inositol treatment, 332 ± 10 g in the treated group vs 300 ± 13 g in the non-treated diabetic group as compared to 381 ± 14.1 g in the non-diabetic-resistant line.

Kidney weight, creatinine clearance and proteinuria

Kidney weight per 100 g/body weight was unaffected by *myo*-inositol treatment, 0.553 ± 0.032 g in the treated vs 0.575 ± 0.379 g in the untreated diabetic animals. They were both significantly higher than in the non-diabetic controls, at 0.347 ± 0.012 g.

Creatinine clearance. Although creatinine clearance is not a good measure of glomerular filtration rate (GFR) in diabetic rats, as significant tubular handling of creatinine occurs, it gave an approximate idea of the renal function when compared with the diabetic controls. Thus, it was lower in the diabetic than in the non-diabetic animals and was unaffected by the treatment being 0.303 ± 0.026 ml · min^{-1} · 100 g body $weight^{-1}$ in the treated, and 0.262 ± 0.0291 in the untreated diabetic group as compared to 0.92 ± 0.046 in the normal non-diabetic resistant line.

Proteinuria. In the non-diabetic rats (group III) protein excretion was 11.9 ± 2.2 mg/24 h. Proteinuria in the diabetic rats was unaffected by *myo*-inositol treatment, being 77.4 ± 22.7 mg/24 h in the untreated diabetic group as compared with 79.1 ± 22.78 in the treated diabetic group.

Na^+-K^+-ATPase

When we compared medullary Na^+-K^+-ATPase activity in animals with and without GS as a marker of the disease and pooling the results of animals with high and low protein in each group, we found that in the treated group I (animals numbers 13 and 14 with GS) it was 49 μmol · mg $protein^{-1}$ · h^{-1} and in animals 9–14 (three with GS) it was 77. In the untreated diabetic group II (animals numbers 9–14, all with GS) it was 146 μmol · mg $protein^{-1}$ · h^{-1} (220 + 72 = 292:2). In group III without GS (animals 1–6) it was 129 μmol · mg $protein^{-1}$ · h^{-1} (127 + 132 = 259:2). In the cortex in the untreated group I (animals 13 and 14) the Na^+-K^+-ATPase was 47 and in animals numbers 9–14 (3 with GS) it was 45 (47 + 42 = 89:2). In the untreated diabetic group II (animals numbers 9–14 all with GS) it was 61 (49 + 72 = 124:2) and in the non-diabetic group III it was 63 μmol · mg $protein^{-1}$ · h^{-1} (49 + 77 = 124:2). It was apparent that in the treated group the medullary and the cortical Na^+-K^+-ATPase activity levels were lower, 49 or 77 μmol · mg $protein^{-1}$ · h^{-1} than 146 found in the untreated diabetics. In the cortex of the treated group it was 47 or 45 vs 61 μmol · mg $protein^{-1}$ · h^{-1} in the untreated diabetic rats. Although these figures cannot be tested for their statistical significance, they represent a pool of six animals each and the difference in the medullary Na^+-K^+-ATPase activity between the treated and untreated diabetic rats is about 50 %.

Renal pathology

In the non-diabetic resistant line, there were no pathological changes in the kidney as previous experience has shown in sucrose-fed resistant animals [19]. In the diabetic animals severe renal pathological changes were observed which remained unaffected by the treatment. Among the kidneys of the 14 treated diabetic animals (group I), seven were grade 3–4^+ GS, one was grade 2^+, while six were normal. Among the kidneys of the untreated diabetic rats (group II) there were nine animals with grade 3–4^+ GS, and five were normal, χ^2 ($df = 2$) = 1,9957. In all rats with very high urinary protein excretion in both groups I and II the pathological changes were grade 3–4+, although not all the animals with severe pathological changes had high urinary protein excretion and vice versa.

Discussion

Hyperglycaemia is an important aetiologic factor in the development of vascular complications in diabetic patients [20–22]. Animal studies have provided direct links between hyperglycaemia and histological changes in the kidney and retina [10, 23]. The exact mechanism of onset of diabetic complications has not been clarified and multiple genetic, metabolic and vascular factors may be involved.

It has been postulated that lower concentrations of intracellular *myo*-inositol as a result of sorbitol accumulation may contribute to the pathogenesis of diabetic nephropathy [1, 2]. This is deducted from short-term studies in streptozotocin diabetic (SZT-D) (IDDM) animals showing that early administration of aldose reductase inhibitors reduces the increased GFR [5, 6] and partly reduces the increased urinary albumin excretion [6–8, 24]. The results of the studies on the effect of dietary *myo*-inositol supplementation on the GFR differ. Goldfarb et al. [25] report a decrease while Cohen et al. [28] did not find a change, although they report a decrease in renal Na^+-K^+-ATPase.

In our study, long-term (4 months) *myo*-inositol supplementation to the Cohen diabetic (type 2) animals did not reduce the increased renal weight and the urinary protein excretion or affect the reduced creatinine clearance, neither did it improve or reduce the renal pathological changes. This is in contrast to the reported beneficial effect of *myo*-inositol dietary supplementation on nerve conduction [26] and the disruption of structural neural elements [27]. A possible explanation for these differences may be that in the nerve tissue, contrary to renal tissue, Na^+-K^+-ATPase [28–30] and *myo*-inositol [29–33] are reduced, whereas in the renal cortex and medulla the Na^+-K^+-ATPase is elevated [15, 28, 33] and the outer medulla is rich in *myo*-inositol [28, 34, 35]. Also, in nerve tissue vasoconstriction and reduced blood supply occur [36–38] while the kidney shows vasodilatation [39, 40] and increased blood flow [41–43]. In the nerve, Na^+-K^+-ATPase impairment results in sodium accumulation, which a) selectively blocks nodal depolarization, thereby diminishing composite conduction velocity [30, 44], and b) leads to the swelling of cells and disruption of structural elements [27]. In the diabetic kidney there is extensive electrolyte loss resulting from increased glucose diuresis and increased glomerular filtration resulting from vasodilatation caused by renal prostaglandin (PG) formation. The increased renal tubular Na^+-K^+-ATPase which causes excessive electrolyte reabsorption [45] may compensate for these physiological changes [46].

The present study emphasizes the difference between the reported beneficial effect of *myo*-inositol supplementation on the increased GFR in the early stages of SZT-D and the lack of effect of long-term *myo*-inositol treatment on the development of severe diabetic nephropathy in the Cohen rat. The precise role of increased glomerular pressure in the evolution of diabetic glomerulopathy is uncertain [47]. In rats, severe diabetic nephropathy can occur without an increase in glomerular capillary pressure [48, 49] and in humans [50–52] hyperfiltration does not appear to be a predictor of the development of proteinuria and renal dysfunction. Also, in the rat acute SZT-D causes natriuresis and increased urinary output that precedes the increase in GFR [15]. The enhanced tubular reabsorption of glucose, Na^+ and water may increase the GFR [53, 54]. The above studies suggest that: a) the GFR in SZT-D may be modified in a way that is independent of the effect of *myo*-inositol on kidney ATPase; b) that even if the GFR is improved by *myo*-inositol treatment it does not necessarily prevent the development of nephropathy in diabetes; and c) as stated above increased GFR in diabetes might be the result of increased glucose diuresis and increased PG synthesis [54, 55], which lead to hyperfiltration [40, 41, 55].

The progression of renal pathological changes in the present study despite the *myo*-inositol treatment, indicates that they may be due to other metabolic and humoral factors resulting from hyperglycaemia, such as: 1) mechanisms other than the aldose reductase pathway, such as altered pyridine nucleotide ratios, which affect other cellular biochemical steps [56]; 2) in diabetes there is increased de novo production of diacylglycerol [57] in the glomeruli [58] and cultured mesangial cells [59] which results in increased protein kinase C activity which causes enhanced growth of vascular cells [60], increased smooth muscle cell contraction [61], and increased cyclic AMP responses to different hormones in vascular cells [62], and increased PG synthesis by glomeruli and mesangial cells [54, 57]. Overproduction of PG plays a role in early renal hyperfusion and hyperfiltration [39, 40, 43, 63]; 3) Excessive formation of free radicals [64] which are cytotoxic to vascular endothelial cells [65] and contribute to the progressive deterioration of function in diabetic tissues [66, 67]. Oxidative modification of LDL is a key step in the formation of foam cells, as such modification is required to allow macrophage receptors to recognize LDL [68]. The analogy between diabetic nephropathy and atherosclerosis has been made previously [69, 70]. In the present model there is a large number of foam cells in the glomeruli (Fig. 1) with a severe degree of GS, which may be due to the oxidative modification of LDL; [2] in the presence of hyperglycaemia non-enzymatic glycation of proteins occurs, which turn slowly into advanced glycation end products. These accumulate constantly on long-lived vessel wall proteins contributing to the thickening of basement membranes [71]. In our previous study [72] repeated injections of glycated plasma resulted in the development of GS in the Cohen prediabetic rats.

Several biochemical disturbances provoked by hyperglycaemia have been implicated in the development of irreversible structural tissue changes in diabetes. In the SZT-D nerve, *myo*-inositol supplementation was reported to restore impaired conduction velocity [26], low Na^+-K^+-ATPase and disruption of structural elements [27] in the nerve and reduce the increased GFR and proteinuria of the early changes of SZT-D. In contrast, long-term administration of *myo*-inositol to the Cohen diabetic rat had no beneficial effect on the development of renal pathological changes although it reduced the elevated renal Na^+-K^+-ATPase activity. These results indicate that the effect of the biochemical disturbances ensuing from hyperglycaemia vary in different diabetic tissues and depend upon local conditions.

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