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KIDNEY SIALIDASE AND SIALYLTRANSFERASE ACTIVITIES IN SPONTANEOUSLY AND EXPERIMENTALLY DIABETIC RATS

INFLUENCE OF INSULIN AND SORBINIL TREATMENTS

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Abstract—Kidney cortex sialic acid level, sialidase and sialyltransferase activities have been measured in spontaneously diabetic BB rats and in streptozotocin-diabetic rats (STZ). In untreated diabetic BB rats, at the onset of the disease, sialidase specific activity was found to be increased by 21% when compared with diabetes-resistant BB controls ($P < 0.05$) whereas sialyltransferase activity was not significantly modified and bound sialic acid concentration was diminished ($P < 0.05$). In diabetic BB rats submitted to a minimal insulin therapy, during 3 months of disease, sialidase activity and sialic acid concentration were similar to those of Wistar age-matched controls. In STZ-diabetic Wistar rats, sialidase specific activity was increased by 76% after 5 months of disease when compared to age-matched Wistar controls ($P < 0.01$); in contrast, specific sialyltransferase activity was decreased by 21% ($P < 0.05$); these enzymatic alterations were associated with a decrease in bound sialic acid concentration ($P < 0.01$); 1 month's insulin therapy, started 4 months after onset of the disease, normalized sialidase activity but had no effect on sialyltransferase activity and sialic acid concentration; treatment with sorbinil prevented cataract development but had no effect on sialidase activity whereas it emphasized the decrease in sialyltransferase activity and sialic acid concentration. The disturbances in the enzyme activities concerned with sialoglycoconjugate metabolism observed in experimental and spontaneous diabetes may be responsible for the decreased bound sialic acid content observed in the rat kidney cortex.

Sialic acids are found in important glomerular basement membrane (GBM†) glycoproteins like laminin, nidogen and procollagen IV contributing to the negative charges of the GBM [1–3]. They also contribute to the polyanionic surface coat of endothelial and epithelial cells of the capillary wall [4, 5]. Cell surface sialic acids appear to be required for the maintenance of the specialized shape of the podocytes [6]. Sialic acids are also present in tubular cells and tubular basement membrane where they can interfere with ionic transport [7, 8]. Together with heparan sulfate proteoglycans, sialic acid may contribute to the charge/size selectivity of the glomerular filtration [9–12]. In diabetes, a loss of sialic acids in GBM [13, 14] and in other tissues has been observed [15–17]. Increased albuminuria and hypoalbuminemia have been described in this disease [18, 19]; they may be explained, at least in part, by the decrease in negative charges of the glomerular capillary wall. Treatment with sorbinil, an aldose reductase inhibitor, has been reported to prevent proteinuria in diabetic rats [18]. On the other hand insulin therapy seems able to reverse increased albumin excretion in diabetic rats [20, 21]. This led us to investigate the effect of sorbinil and insulin on the activity of two enzymes concerned with sialic acid metabolism: *sialidase* (EC 3.2.1.18) and *sialyltransferase* (EC 2.4.99.1). In a previous paper, we have shown that specific and total sialidase activities were increased in rat kidney cortex dialysed homogenates after 80 days of streptozotocin-induced (STZ) diabetes [22]. In this work, we have extended the study to include kidney cortex sialyltransferase activity and sialic acid level modifications and in addition studied all three parameters in another diabetic model, the spontaneously diabetic BB rat [23].

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† Abbreviations: STZ, streptozotocin; BB, Bio Breeding; GBM, glomerular basement membrane; Cytosine monophosphate *N*-acetyl neuraminic acid, CMPNeu5Ac.

MATERIALS AND METHODS

Spontaneously diabetic Bio Breeding Wistar (BB) rats

Spontaneously diabetic BB rats were obtained from the Animal Resources Division, Health and Welfare, Ottawa, Canada (Dr P. Thibert).

Diabetic rats at onset of the disease. Male prediabetic BB rats derived from the mating between the diabetic male and the diabetic or nondiabetic female were tested daily for glycosuria, after the age of 8 weeks. Ten animals who had developed diabetes between 11 and 15 weeks of age were killed by decapitation 1 or 2 days following the appearance of glycosuria without any insulin treatment (DBB).

Ten age-matched normoglycemic rats from a diabetes-resistant control colony, issuing from a selective breeding of the same original colony as the diabetic BB rats, were used as controls (CBB).

Three months diabetes. Untreated spontaneously diabetic BB rats cannot survive and rapidly die in ketoacidosis. Therefore male spontaneously diabetic BB rats (N = 11) were treated with an individually adjusted daily subcutaneous injection of protamine zinc bovine insulin started on the time of the first positive glycosuria test (8–14 weeks after birth). Minimal insulin doses (about 8 units/kg body wt) were chosen in order to maintain survival of the animals with 3+ glycosuria. When killed, diabetic BB rats were between 5 and 6 months old (DBB + I). No mortality was observed in this group. These animals were compared with normal control (C) 5-month-old Wistar rats (N = 8, IFFA CREDO, L'Arbresle 69210 France) since age-matched diabetes-resistant BB rats were not available.

Streptozotocin-diabetic rats

Three months diabetic. Eighteen male 9-week-old Wistar rats, IFFA CREDO (250 ± 8 g, SD) were divided into two groups. One group (N = 10) was injected intramuscularly, at day 0 and day 7, with streptozotocin (Sigma Chemical Co., St Louis, MO) 55 mg/kg body weight in 100 mM sodium chloride, 1 mM citrate buffer pH 4.5. The age-matched control group (N = 8) was injected with buffer alone. Animals were housed 4–6 to a cage and allowed free access to food (Standard pelleted rat chow A 103, UAR Co., Villemoisson, France) and water. Diabetic and control rats were killed alternately by decapitation 3 months after the induction of diabetes. No mortality was observed in these groups.

Five months diabetes. Sixty-nine male 7-week-old Wistar rats, IFFA-CREDO (170 ± 5 g, SD) were divided in four groups. Two groups were injected with streptozotocin and two groups with buffer alone as above. One of the diabetic groups (N = 12) and control groups (N = 8) were treated daily from day 1 until killing with sorbinil (12 mg/kg body wt dissolved in 0.5% carboxymethylcellulose) through oesophageal cannula. The other control group (N = 16) and diabetic group (N = 33) were treated with vehicle alone. In the latter diabetic group, a subgroup (N = 9) was treated daily with a subcutaneous injection of protamine zinc bovine insulin started after 4 months of diabetes, (20 units/kg on the first days, then adjusted individually in order to approach normal blood glucose level). During the experiment, one animal died in the diabetic untreated group (finally N = 23), two in the insulin-treated diabetic group (finally N = 7) and none in the other groups.

Cataract

Cataract was evaluated by clinical inspection and ophthalmoscopic examination. Cataract was considered positive when clinically evident for an external physician.

Enzymatic determinations

Kidney fractions. Immediately after killing, the right kidney cortex was isolated and homogenized with a potter homogenizer in cold 75 mM sodium acetate buffer pH 4 (4 mL/g cortex); half the volume of each homogenate was extensively dialysed against the same buffer. Sialidase activity was promptly assayed in these freshly prepared homogenates. Aliquots were preserved for sialic acid determinations. The left kidney was frozen in liquid nitrogen and kept at −80° until used for sialyltranserase determinations. After thawing, the cortex was removed and homogenized with a Teflon pestle in 0.1 M Tris–HCl pH 6.8 buffer containing 0.1% Triton X100 and 2 mM β-mercaptoethanol (4 mL/g cortex). The homogenates were subjected to seven brief ultrasonic series at an amplitude of 2 emitted from a Branson Sonifier. The homogenate was centrifuged at 10,000 *g* for 25 min and the supernatant was used as enzyme source.

Assay for sialidase activity. This was effected according to the method of Frish and Neufeld [24] with slight modifications. The incubation system contained: 100 μL of dialysed or non-dialysed fresh kidney cortex homogenates (approx. 150 μg protein); 0.6 nmole (25 Ci/mol) of sialylα2-3[^{3}H]lactitol isomer as substrate purified and radiolabelled as previously described [5]; 50 mM sodium acetate buffer pH 4 and 3 mM sodium azide in a final volume of 150 μL. The incubations were carried out at 37° for 10–20 min. Under these conditions, linearity was found with enzyme concentration and time. In order to evaluate the influence of the endogenous substrates upon sialidase activity toward sialylα2-3[^{3}H]lactitol, 150 μg of heat-inactivated proteins (5 min at 80°) were added to the incubation assay. Only 8% inhibition of sialidase activity was observed.

Assay for sialyltransferase activity. Assays were carried out according to Bardos *et al.* [25] with slight modifications: 80 μL aliquots containing about 0.2 mg protein of the enzyme preparation were incubated at 30° for 30 min with 20 μL of the 0.2 M Tris–HCl pH 6.5 buffer containing 0.1 M $MnCl_2$ and 0.1% Triton X100, 10 μL of asialofetuin (50 mg/mL, Sigma) and 0.6 nmole cytosine monophosphate [^{14}C]-*N*-acetyl neuraminic acid, 33 Ci/mol (CMPNeu5Ac, Amersham International, Amersham, U.K.). The same mixture omitting the exogenous substrate served for the evaluation of the enzyme activity toward endogenous acceptors. The reaction was stopped by addition of 1 mL cold 1% (w/v) phosphotungstic acid in 0.5 M HCl. The acid precipitable material was sedimented by centrifugation at 3000 *g* for 10 min and washed twice with cold 10% (w/v) trichloroacetic acid. The pellet was then washed with 1 mL ethanol–ether (2:1 v/v), dried and dissolved in 0.2 mL NaOH 0.5 M and 2.5 mL of picofluor added. The radioactivity was measured using an Intertechnique SL3 000 liquid scintillation counter (Beckman). The amount of radioactive sialic acid incorporated was expressed in cpm/hr/mg protein (after subtraction of the radioactivity incorporated into the endogenous acceptors, about 2%). Under these conditions, linearity was observed with enzyme concentration and time.

Chemical determinations

Total and bound kidney cortex sialic acid concentrations were determined in non-dialysed and dialysed homogenates after hydrolysis in 0.05 M

Table 1. Characteristics of the spontaneously diabetic rats and their controls

	Stage of diabetes at onset		after 3 months	
	BB diabetic (N = 10)	BB control (N = 10)	BB diabetic + I (N = 11)	Wistar control (N = 8)
Body weight (g)	302 ± 11*	367 ± 17	440 ± 12	428 ± 8
Plasma glucose (mM)	30.4 ± 1.3†	6.9 ± 0.3	25.2 ± 2.1†	5.7 ± 0.2
Plasma fructosamine (nM)	ND	ND	2.24 ± 0.09†	1.71 ± 0.08
Kidney weight (g)	1.37 ± 0.05	1.47 ± 0.06	1.55 ± 0.06‡	1.14 ± 0.03
Kidney cortex (% kidney weight)	83.2 ± 4.4	85 ± 3	87.7 ± 4.9	85 ± 3
Kidney cortex proteins (mg/g cortex)	159.6 ± 4.1	151 ± 4.1	183.8 ± 4.4	170 ± 5.6

Values are means ± SE.
* $P < 0.05$; † $P < 0.01$; ‡ $P < 0.001$ versus corresponding normoglycemic controls.
ND, not determined; I, insulin.

H_2SO_4 at 80° for 1 hr using the thiobarbituric reagent [26]. Prior to sialic acid determination, hydrolysed samples were passed through AG1X2 columns to remove interfering chromogens. All determinations were made using a *N*-acetyl neuraminic acid standard (Sigma) treated in the same way. The same steps were applied for determinations of sialic acids released by kidney sialidase from endogenous substrates. Total protein concentrations were measured by the method of Lowry *et al.* [27]. Aliquots of homogenates were solubilized first in 0.1 M NaOH at 37° for 2 hr. Plasma glucose concentrations were measured by a micro-glucose oxidase techniques [28]. Plasma fructosamine levels were measured with a fructosamine test kit ROCHE. Albumin was determined in plasma using electroimmunodiffusion.

Statistical methods

Results are expressed as means ± SE. In the experiments with two groups, the statistical difference between mean values was evaluated by Student's *t*-test. Only when a variance difference was found by the F test, the comparison was carried out by Mann-Whitney's U test. In the 5 months STZ-diabetes experiment with four groups, the influence of diabetes or sorbinil treatment on the various parameters was studied by two-way variance analysis. The insulin-treated group was individualized and compared by one-way variance analysis to the untreated diabetic group and to the untreated normoglycemic group. Comparisons between two groups were then effected by Student's *t*-test, corrected for multiple comparisons using the residual variance. Aberrant values were detected by the maximum deviation from the mean test. The significance threshold retained was $P < 0.05$. After statistical analysis, results were plotted as relative values compared with the respective normoglycemic control group.

RESULTS

Characteristics of the experimental animals

The characteristics concerning the untreated diabetic BB rats at onset of the disease and the poorly controlled long-term diabetic BB rats are presented in Table 1. Body weights of the BB rats at onset of the disease were significantly lower than those of their controls. In contrast, in the long-term diabetic BB rats treated by minimum insulin doses, body weights were not significantly different from those of the normal Wistar controls. No cataract was observed in both groups of BB diabetic rats. The blood glucose level at the time of killing was 30.4 ± 1.3 mM in the untreated BB rats at onset of diabetes and 25.2 ± 2.1 mM in the poorly controlled long-term diabetic BB rats.

The characteristics of the STZ-diabetic rats and their controls are summarized in Table 2. All untreated diabetic rats had reduced body weight, cataract and hypoalbuminemia. Sorbinil treatment prevented cataract development and was effective on hypoalbuminemia without any significant influence on body weight, plasma glucose and fructosamine levels. Curative treatment by insulin, for 1 month, did not reverse the development of cataract which was already present in 93% of the animals at the beginning of the treatment, but it significantly increased body weight and corrected hypoalbuminemia. Blood plasma glucose level was normalized in the insulin-treated group.

Kidney cortex enzymes activities and sialic acid content

Kidney weight. Increase in kidney weight was observed in all STZ-diabetic rats without any effect of sorbinil or insulin treatments (Table 2). In the minimally insulin-treated BB rats with 3 months diabetes this increase was also observed whereas in the untreated newly diabetic BB rats, no difference in the absolute kidney weight was seen at onset of the disease; however, relative to body weight, the kidney weight was increased (Table 1).

Sialidase activity in non-dialysed homogenates (Fig. 1). In the untreated diabetic BB rats, at onset of the disease, the specific sialidase activity was increased by 21% ($P < 0.05$). In the minimally insulin treated long-term diabetic BB rats no difference was observed with that of normoglycemic controls. In STZ-diabetic rats after 3 and 5 months of diabetes, sialidase specific activity was markedly increased (by 90%, $P < 0.01$ and by 76%, $P < 0.001$, respectively).

Table 2. Characteristics of the STZ-diabetic rats and their controls

	Duration of diabetes: Three months		Duration of diabetes: Five months				
	Diabetic (N = 10)	Control (N = 8)	Diabetic (N = 23)	Diabetic + insulin (N = 7)	Control (N = 16)	Diabetic + sorbinil (N = 12)	Control + sorbinil (N = 8)
Body weight (g)	279 ± 12†	428 ± 8	239 ± 9‡	333 ± 7‡‖	462 ± 12	263 ± 3‡	488 ± 12
Cataract (%)	100	0	100	93	0	0	0
Albuminemia (g/l)	ND	ND	30.1 ± 1.0‡	35.7 ± 2.1§	37.1 ± 1.3	34.0 ± 1.1§	37.8 ± 1.8
Plasma glucose (mM)	36.4 ± 0.1‡	5.7 ± 0.2	41.7 ± 1.2‡	6.2 ± 1.9‖	5.9 ± 0.2	38.6 ± 1.7‡	5.8 ± 0.2
Plasma fructosamine (nM)	2.95 ± 0.08‡	1.71 ± 0.08	2.56 ± 0.09‡	1.03 ± 0.18‡‖	1.68 ± 0.06	2.89 ± 0.15‡	1.77 ± 0.12
Kidney weight (g)	1.47 ± 0.03‡	1.14 ± 0.03	1.19 ± 0.06‡	1.16 ± 0.04*	1.00 ± 0.03	1.23 ± 0.04†	1.08 ± 0.03
Kidney cortex (% kidney weight)	82 ± 1	85 ± 3	87 ± 2	89 ± 3	88 ± 3	87 ± 2	88 ± 3
Kidney cortex proteins (mg/g cortex)	164 ± 4.3	170 ± 5.6	143 ± 2.6*	164 ± 5.6*‖	151 ± 2.6	151 ± 2.7	156 ± 3.5

Values are means ± SE (except for cataract incidence).

* $P < 0.05$; † $P < 0.01$; ‡ $P < 0.001$ versus corresponding normoglycemic controls. § $P < 0.05$; ‖ $P < 0.001$ versus untreated STZ-diabetic rats.

ND, not determined.

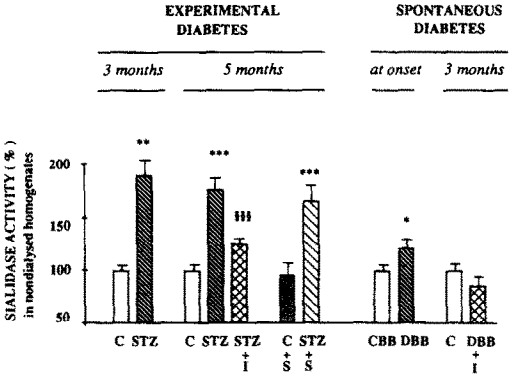

Fig. 1. Kidney cortex sialidase activities in spontaneously and experimentally diabetic rats: influence of insulin and sorbinil treatments. C, untreated Wistar normoglycemic controls; STZ, streptozotocin-diabetic rats; CBB, normoglycemic control BB rats; DBB, diabetic BB rats; I, insulin; S, sorbinil. Sialidase specific activities (means ± SE) are represented as per cent of their respective untreated normoglycemic controls: Wistar controls of 3 or 5 months STZ-diabetes and normoglycemic control BB rats. In nondialysed homogenates the activities, expressed as [^{3}H]lactitol released from sialylα2,3[^{3}H] lactitol in these untreated controls, are respectively: 1080 ± 41 (N = 8); 979 ± 43 (N = 16); 356 ± 19 (N = 8) dpm/min/mg protein. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ versus untreated normoglycemic control rats, the "* symbol" reflecting an effect of diabetes. §§§ $P < 0.001$ versus untreated STZ-diabetic rats, the symbol "§" reflecting an effect of treatment.

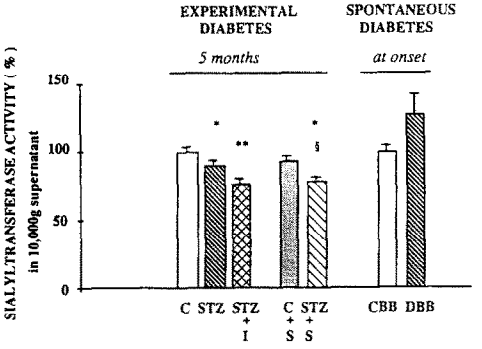

Fig. 2. Kidney cortex sialyltransferase activities in spontaneously and experimentally diabetic rats: influence of insulin and sorbinil treatments. C, untreated Wistar normoglycemic controls; STZ, streptozotocin diabetic rats; CBB, normoglycemic control BB rats; DBB, diabetic BB rats; I, insulin; S, sorbinil. Sialyltransferase specific activities (means ± SE) are represented as per cent of their respective untreated normoglycemic control: Wistar controls of 5 months STZ-diabetes and normoglycemic control BB rats. In these untreated controls, the activities expressed as [^{14}C]CMPNeu5Ac incorporated in asialofetuin are respectively: 3447 ± 174 (N = 16); 3524 ± 250 (N = 10) cpm/hr/mg protein. * $P < 0.05$; ** $P < 0.01$ versus untreated normoglycemic controls, the "* symbol" reflecting an effect of diabetes. § $P < 0.05$ versus untreated STZ-diabetic rats, the "§ symbol" reflecting an effect of treatment. The enzyme activity was not determined in STZ and BB rats after 3 months of diabetes.

One month of insulin treatment corrected this alteration ($P < 0.001$). No effect of sorbinil treatment on sialidase activity was found.

Sialidase activity in dialysed homogenates. Similar results were obtained as with non-dialysed homogenates: thus small molecular weight and dialysable effectors cannot account for the increase in sialidase activity.

Sialyltransferase specific activity (Fig. 2). Sialyltransferase specific activity was not significantly modified in the BB rats on onset of diabetes whereas it was decreased in STZ-diabetic rats after 5 months of diabetes ($P < 0.05$). Neither insulin nor sorbinil treatment reversed this alteration. On the contrary sorbinil treatment further decreased sialyltransferase activity in STZ-diabetic rats ($P < 0.05$).

Sialic acid concentrations (Fig. 3). Total sialic acid concentrations were significantly lowered in the

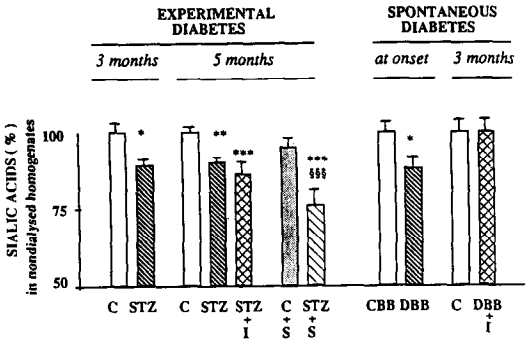

Fig. 3. Kidney cortex sialic acid concentration in spontaneously and experimentally diabetic rats: influence of insulin and sorbinil treatments. C, untreated Wistar normoglycemic controls; STZ, streptozotocin diabetic rats; CBB, normoglycemic control BB rats; DBB, diabetic BB rats; I, insulin; S, sorbinil. Total sialic acids measured in non-dialysed homogenates were expressed as % of their respective untreated normoglycemic controls: Wistar controls of 3 or 5 months STZ-diabetes and normoglycemic control BB rats. Values, in these untreated controls are respectively: 11.7 ± 0.2 ($N = 8$); 11.5 ± 0.2 ($N = 16$); 11.8 ± 0.3 ($N = 10$) nmol NeuAc/mg protein. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ versus untreated normoglycemic controls, the "* symbol" reflecting an effect of diabetes. §§§ $P < 0.001$ versus untreated STZ-diabetic rats, the "§ symbol" reflecting an effect of treatment.

untreated BB rats at onset of diabetes but not different from the Wistar controls in minimally insulin-treated long-term diabetic BB rats. Sialic acid level was also significantly decreased in all groups of STZ-diabetic animals. Insulin did not reverse the alteration. Sorbinil further decreased sialic acid concentration in diabetic rats. Bound kidney cortex sialic acids represented 97–99% of total kidney cortex sialic acids.

DISCUSSION

Kidney cortex sialidase specific activity was found here to be increased in spontaneously diabetic BB rats as in STZ-diabetic rats. This alteration is observed at early diabetic stage (1–2 days post detection of glycosuria) in the BB rats and may be responsible for the decrease in sialic acid level. Insulin deficiency due to autoimmune insulitis has been demonstrated to be responsible for the clinical onset of diabetes in BB rats [23, 29, 30]. Three months of minimal insulin treatment started at onset of the disease in the BB rats resulted in normalization of sialidase activity and sialic acid level. On the other hand, in STZ diabetic rats which are known to be insulin deficient [30], 1 month of insulin treatment started 4 months after the induction of diabetes resulted also in normalization of sialidase activity but not in sialic acid level. This is compatible with a determinant role of insulin deficiency in the genesis of the rise in sialidase activity. Insulin deficiency rather than hyperglycemia might be responsible for the alteration since sialidase activity is normal in minimally insulin-treated BB rats in spite of hyperglycemia and increased fructosamine level at sacrifice. The failure of insulin treatment to improve sialic acid alteration in STZ-diabetic rats may be due to the persistent decrease of sialyltransferase specific activity under this treatment. The results obtained with the two diabetic models showed that the alteration in sialidase activity and/or in sialyltransferase activity could lead to a decrease in kidney cortex sialic acid level. Indeed a proper balance between biosynthetic and degradative pathways seems to be required for the regulation of normal sialylation level of the glycoconjugates.

Sorbinil treatment appears to further decrease kidney sialyltransferase activity and sialic acid level in STZ-diabetic rats without changing sialidase activity. Thus, the effect on sialic acid level seems related to the decrease in sialyltransferase activity. Sorbinil emphasizes the effect of diabetes in spite of its beneficial effect on cataract development and hypoalbuminemia observed here, and on vascular reactivity alterations studied in the same animals [31]. The effect of sorbinil on hypoalbuminemia is consistent with its corrective effect on proteinuria reported by Beyer-Mears *et al.* [18]. The lack of beneficial effect of sorbinil on anionic charges has also been reported for heparan sulfate [32].

Our results concerning the increased sialidase activity in kidney cortex of STZ-diabetic rats are in agreement with those of Metzger *et al.* [33] briefly reporting an increase of the enzyme activity in liver and kidney of STZ-diabetic rats. As far as sialyltransferase is concerned, Bardos *et al.* [34] have described a decreased kidney cortex specific activity in 13-month-old diabetic db/db mice but not in 4-month-old animals. Moreover, a reduced synthesis of CMP sialic acid, one of the substrates of sialyltransferase, has been found in the kidney cortex of diabetic South African hamster [35]. Thus, the decrease in bound sialic acid may be due to modifications of several enzyme activities concerned with the metabolic pathway of sialoglycoconjugates. This may result in functional abnormalities of various nephron structures: contribution to increased charge or size selective glomerular permeability, altered ion or glucose tubular transport, activation of the alternative complement pathway [36, 37]. The elevated sialidase activity may be further associated with lysosomal abnormalities: increased degradation in kidney tubular lysosomes of reabsorbed sialoglycoconjugates like plasma α_1 acid glycoprotein [17], increased degradation of other sialylated lysosomal enzymes such as acid phosphatase whose activity is decreased in diabetic tissues [38].

In conclusion, sorbinil has no corrective effect on sialic acid metabolism alterations. On the other hand, insulin corrects the alteration of sialidase activity but not the decrease in sialyltransferase activity and sialic acid level. Thus, the beneficial effect of sorbinil and insulin on hypoalbuminemia does not appear to be mediated through a correction of sialic acid level but could be related to other effects, particularly on hemodynamic parameters.

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