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Diabetes mellitus induced inhibition of glucosaminyl N-deacetylase: effect of short-term blood glucose control in diabetic rats

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Summary. Inhibition of glucosaminyl N-deacetylase activity, a key enzyme in heparan sulphate sulphation, may be involved in the development of late diabetic vascular complications. We examined the effect of short- and long-term metabolic control on N-deacetylase activity in streptozotocin diabetic H and U rats. Spontaneously diabetic BB rats were included in parts of the study. Over a 3-week period blood glucose was maintained at predetermined levels (6–10 mmol/l or 10–20 mmol/l) by insulin treatment and then during the final 2 days rapidly reversed in half of each group. In the U rats, the *hepatic* N-deacetylase activity significantly decreased by 10–15% following short-and long-term poor metabolic control and the inhibition was entirely reversed by short-term good control. In the H rats a similar, not significant, effect was seen. BB rats in long-term poor control showed a 10% reduction in hepatic N-deacetylase activity ($p = 0.003$). *Glomerular* N-deacetylase activity was reduced in U rats after long-term poor control ($p = 0.004$) but not in H and BB rats. There was an overall correlation between urinary albumin excretion and glomerular N-deacetylase activity ($r = -0.60, p < 0.0001$). We conclude that diabetes-induced inhibition of hepatic N-deacetylase is not restricted to the streptozotocin diabetic model, and that short-term blood glucose control is of major importance. Genetic factors and tissue specificity influence the vulnerability of the enzyme. Finally, the study suggests an association between N-deacetylase activity and urinary albumin excretion.

Key words: Heparan sulphate, experimental diabetes, BB rat, albuminuria.

Impairment of heparan sulphate biosynthesis may have a central role in the pathogenesis of diabetic vascular complications [1–4]. Diabetes-induced inhibition of a key enzyme in heparan sulphate glycosaminoglycan synthesis, glucosaminyl N-deacetylase (EC no. 3.5.1.33), [5–7] leads to synthesis of low-sulphated heparan sulphate molecules [8], which may in turn cause the glomerular mesangial expansion, increased permeability, progression of glomerulosclerosis [9–12], and increased atherogenesis as seen in patients who develop diabetic nephropathy [13, 14]. It has been hypothesized that genetic factors related to the vulnerability of the N-deacetylase may provide a clue to the apparent differences in individual risk of developing vascular late diabetic complications [4].

The mechanism of the N-deacetylase inhibition is virtually unknown, and the duration of poor metabolic control required to produce the inhibition has not been settled. Such information would obviously be of help in identifying potential mechanisms of the inhibition. Furthermore, we have previously suggested that genetic factors may cause differences in the vulnerability of N-deacetylase towards short-term metabolic control [7]. However, the previous study did not include intended manipulation of short-term metabolic control and the suggested role for genetic factors therefore needs to be validated. Finally, diabetes-induced N-deacetylase inhibition has up to now been studied in the streptozotocin diabetic rat model only. Although there is strong evidence that the inhibition is associated with the diabetic state per se [5, 7], the use of other diabetic models are needed to certify this association.

In the present study we have therefore examined the time course relationship between metabolic control and inhibition of the N-deacetylase activity in streptozotocin diabetic rats, as well as the effect of diabetes on N-deacetylase activity in spontaneously diabetic BB rats. The potential role of genetic factors in governing the vulnerability of the enzyme were primarily evaluated by including the two Sprague Dawley rat strains also used in previous studies [5, 7]. In addition, since the BB rat is known to be less prone to development of diabetic albuminuria and renal morphological changes [15], a potential association between this trait and reduced vulnerability of the N-deacetylase were also explored.

Materials and methods

Design: Adult (3-month-old) male H and U Sprague-Dawley rats, and spontaneously diabetic BB rats. H rats are standard Sprague-Dawley rats (from Zentralinstitut für Verzuchstiere, Hannover, FRG). The U rats originate from the Hannover strain but since 1962 they have been bred separately in Uppsala, Sweden, currently at the University of Uppsala, Department of Medical Cell Biology [16]. BB rats originated from the inbred BB-Hagedorn strain (DP BB/Hrl) [17]. In H and U rats, diabetes was induced by i. v. injection of streptozotocin (Sigma S-0130, St.Louis, Mo., USA) 55 mg/kg in 0.05 mol/l citrate buffer ph 4.2, after overnight fasting. Only animals with blood glucose above 15 mmol/l measured 2 days after the streptozotocin injection were included. BB rats were included at onset of diabetes. All animals were insulin treated (H and U: one daily injection; BB rats: two daily injections, s.c. of Ultra Lente bovine heat-treated insulin; Novo Nordisk, Bagsvaerd, Denmark) and randomized to blood glucose control in the range 6–10 mmol/l (group D1, "good control") or 10–20 mmol/l (group D2, "poor control"). Insulin dose was 12 ± 3 IU/kg in D1 and 6 ± 2 IU/kg in D2 during the period of stable control. Free access was allowed to water and standard fodder. Morning blood glucose was measured daily. After 2 weeks a 24-h urine specimen was measured for urinary albumin excretion rate. After 3 weeks the animals were killed by decapitation. Two days before termination of the study, metabolic control was shifted from good to poor and poor to good by adjusting the insulin dose in half of the H and U rats from the groups D1 and D2, respectively (groups $D1_{\rightarrow 2}$ and $D2_{\rightarrow 1}$). We included non-diabetic H and U rats as controls.

Sample preparation: The ventromedial liver lobe was removed, quick-frozen on dry ice and stored at –80°C until isolation of the microsomal hepatocyte fraction as previously described [5]. Both kidneys were then removed, allowed to bleed out and weighed after removal of the perirenal capsule. Approximately 0.5 g kidney cortex was quick-frozen and stored for the mRNA assay as described below. Glomeruli were isolated from the remaining kidney cortex by sequential sieving and the glomerular microsomal fraction prepared as previously described [7].

N-deacetylase assay: The assay essentially followed the method of Navia et al. [18], using N-[^{3}H]acetyl labelled *Escherichia coli* K5 capsular polysaccharide as substrate and was performed as described previously [7]. A substrate concentration of 5 mg/l was used for both liver and glomerular microsomal preparations and the amount of protein in the samples was in the range of 0.1 to 0.3 mg. The N-deacetylase activity is expressed as cpm · mg total protein^{-1} . 30 min^{-1} . The within- and between-assay coefficients of variation were 4% and 10%, respectively. Glomerular N-deacetylase activity was not measured in the samples from seven animals (2 H (1 D1, 1 $D1_{\rightarrow 2}$), and 5 U (3 D1, 2 $D1_{\rightarrow 2}$), which were lost during preparation).

Urinary albumin: Urinary albumin was measured with a 4-layer sandwich enzyme linked immunoassay based on polyclonal rabbit anti-rat-albumin (RARa/alb; Nordic, Tilburg, The Netherlands) [7].

mRNA Dot blot analysis: Total RNA was isolated from kidney cortex by the method of Chromczynski and Sacchi [19]. RNA yields were determined spectrophotometrically. Dot blots onto nitrocellulose membranes were made from serial dilutions of RNA using a Schleicher and Schuel (Dassel, FRG) Minifold apparatus as follows: 40 µg of total RNA was adjusted to 10% formaldehyde 10× SSC (1× SSC = 0.15 mol/l NaCl, 0.015 mol/l sodium citrate). Four 1: 1 serial dilutions were then made diluting with the same buffer containing tRNA carrier. Of each sample, 10, 5, 2.5 and 1.25 µg RNA were then vacuum blotted onto nitrocellulose (Schleicher and Schuel) equilibrated in 20× SSC. The wells were rinsed in 20×

Table 1. Characteristics of the treatment groups

	H					U					BB	
	C	D1	$D1_{\rightarrow 2}$	D2	$D2_{\rightarrow 1}$	C	D1	$D1_{\rightarrow 2}$	D2	$D2_{\rightarrow 1}$	D1	D2
n	5	9	8	9	3	5	9	8	9	9	9	9
3-week mean BG[a] (mmol/l)	–	8.4 ± 1.0	8.5 ± 1.0	15.6 ± 1.4	16.6 ± 1.5	–	8.2 ± 1.1	8.5 ± 0.7	16.7 ± 1.4	15.7 ± 1.3	9.0 ± 0.8	17.2 ± 1.3
24-h mean BG[a] (mmol/l)	–	5.2 ± 1.7	15.2 ± 3.3	16.3 ± 3.2	11.6 ± 3.2	–	5.9 ± 2.2	14.2 ± 1.8	14.9 ± 1.5	7.4 ± 2.3	7.2 ± 3.5	15.6 ± 3.1
Final BG[a] (mmol/l)	5.8 ± 0.2	5.8 ± 3.6	17.6 ± 3.5	16.2 ± 5.1	11.4 ± 1.3	4.4 ± 0.2	7.5 ± 3.0	17.1 ± 2.1	14.2 ± 2.7	6.2 ± 3.2	8.5 ± 5.7	15.9 ± 6.0
Kidney weight (g/kg body weight)	6.0 ± 0.5	6.2 ± 0.3	6.5 ± 0.4	7.5 ± 0.4	7.1 ± 0.5	5.8 ± 0.4	5.2 ± 0.3	5.8 ± 0.3	6.4 ± 0.5	6.2 ± 0.4	6.3 ± 0.3	7.3 ± 0.6
Glomerular protein (µg/10^3 glomeruli)	34 ± 6	35 ± 7	40 ± 14	38 ± 7	28 ± 2	44 ± 16	34 ± 9	29 ± 9	36 ± 9	28 ± 11	38 ± 18	33 ± 9
Number of glomeruli (10^3)	34 ± 3	33 ± 5	32 ± 4	30 ± 8	33 ± 5	25 ± 4	22 ± 6	25 ± 4	30 ± 5	30 ± 4	28 ± 5	29 ± 7
Urinary albumin excretion (mg/24 h)	1.7 (0.9–4.2)	1.1 (0.3–3.7)		3.2 (0.4–13)		12 (5.8–28)	4.3 (1.0–27)		5.8 (1.4–50)		0.3 (0.2–0.7)	0.3 (0.1–0.4)

[a] Blood glucose (BG) (3 week: mean of daily measurements during 3 weeks; 24 h: mean of three measurements during the day before the study was terminated; final: at the time of killing.
C; Non-diabetic; D1, diabetic, BG 6–10 mmol/l for 3 weeks; D2, diabetic BG 10–20 mmol/l for 3 weeks; $D1_{\rightarrow 2}$, BG as D1 followed by 2 days as D2; $D2_{\rightarrow 1}$, BG as D2 followed by 2 days as D1. Values are mean ± SD or median (range). Significant differences are outlined in text (results)

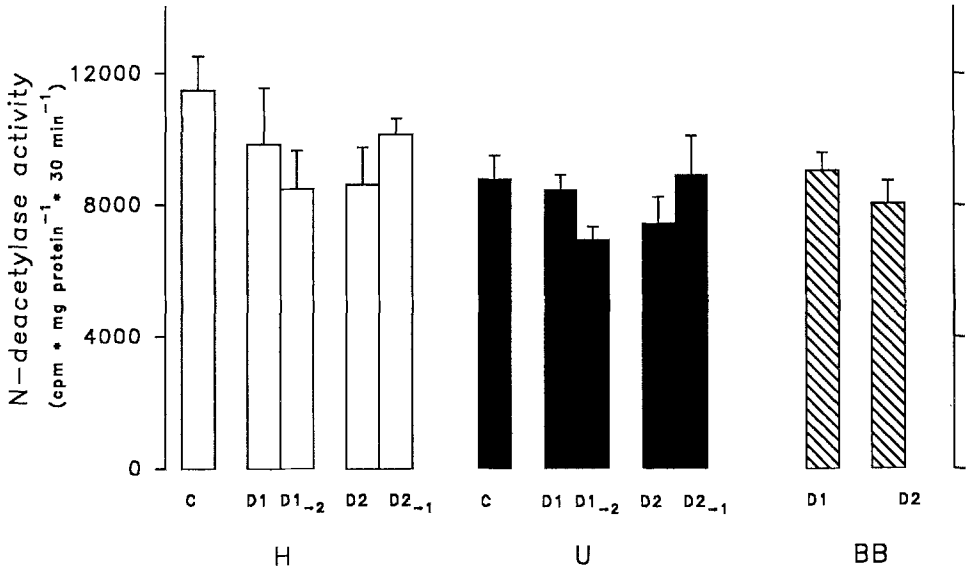

Fig. 1. Hepatic N-deacetylase activity (mean ± SD) in diabetic rats in relation to short- or long-term metabolic control. Three strains (H,U and BB) were studied. C: Non-diabetic; D1: diabetic, blood glucose 6–10 mmol/l for 3 weeks; D2: diabetic, blood glucose 10–20 mmol/l for 3 weeks; D1$_{\rightarrow 2}$: blood glucose as D1, followed by 2 days as D2; D2$_{\rightarrow 1}$: blood glucose as D2, followed by 2 days as D1. Two-tailed p-values (ANOVA): H rats $p = 0.002$ (all), $p = 0.08$ (diabetic only); U rats $p < 0.0001$ (all), $p < 0.0001$ (diabetic only); BB $p = 0.003$

SSC, the blots dried and baked for 2 h at 80 °C under vacuum. Blots were hybridized and washed under stringent conditions as previously described [20]. cDNA clones were labelled by random priming (Boehinger Mannheim, Mannheim, FRG). Clones used were BPG5, a 2 kb fragment encoding a portion of Perlecan [21], and a 0.6 kb Pst I – Eco RI fragment of the p1 B15 clone containing the cyclophillin sequence [22]. After washing, the filters were exposed to Kodak XOmat at –80 °C with intensifying screens. The autoradiograms were scanned for determination of optical density using the Bio Image Whole Band Analysis application. Assignments of relative mRNA values were determined from the 5 µg dot under the condition that: (1) the correlation coefficient in a linear regression of the staining intensity vs RNA amount was above 0.90; (2) 260/280 nm optical density ratio of the RNA was in the range 1.7–2.2 [23]. Samples from four animals (three U, one BB) did not meet these conditions.

Other measurements: Total protein (BCA Protein Assay Reagent (Pierce, Ba-Oud-Beijerland, The Netherlands), using human reference serum (Orion Diagnostics, Espoo, Finland) as standard). Blood glucose was measured on a Cobas Mira automated analyser (Roche, Hvidovre, Denmark)

Statistical analysis

Parametric tests (analysis of variance, Duncan's multiple range test, correlation analysis and multiple linear regression) were used after transformation of not normally distributed variables (urinary albumin excretion). Data were analysed using the StatGraphics version 5.0 software package. Level of significance was 5%, two-tailed. Results are expressed as mean ± SD.

Results

Metabolic control: Blood glucose values are shown in Table 1. The intended levels of metabolic control were accomplished in all groups except in H-D2$_{\rightarrow 1}$, where blood glucose was elevated compared to H-D1 during the final 30 h. Body weight was lower in the BB rats (330 ± 42 g) compared to H and U (473 ± 36 g) but did not differ between treatment groups.

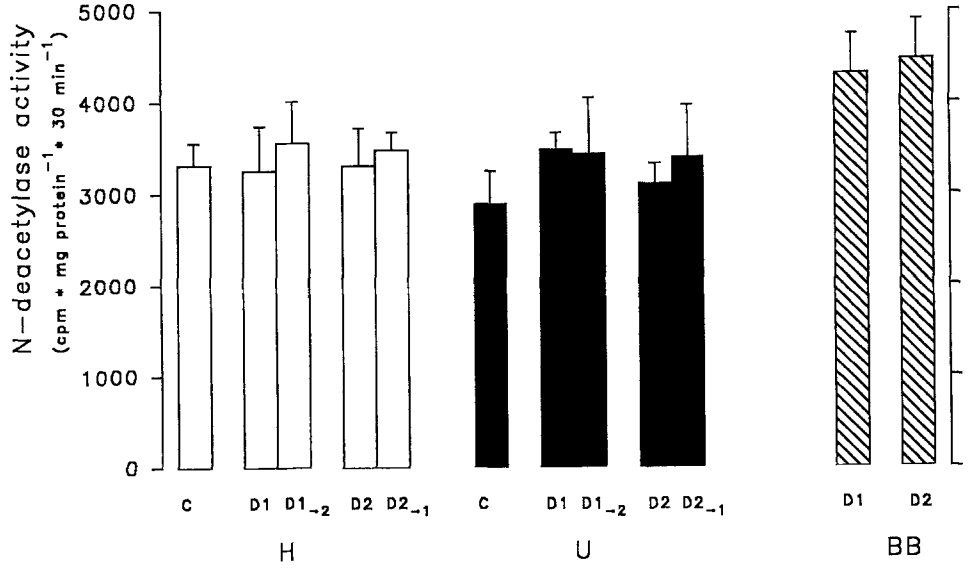

Fig. 2. Glomerular N-deacetylase activity (mean ± SD) in diabetic rats in relation to short- or long-term metabolic control. Two-tailed p-values (ANOVA): H rats $p = 0.6$ (all), $p = 0.5$ (diabetic only); U rats $p = 0.1$ (all), $p = 0.3$ (diabetic only); BB $p = 0.5$

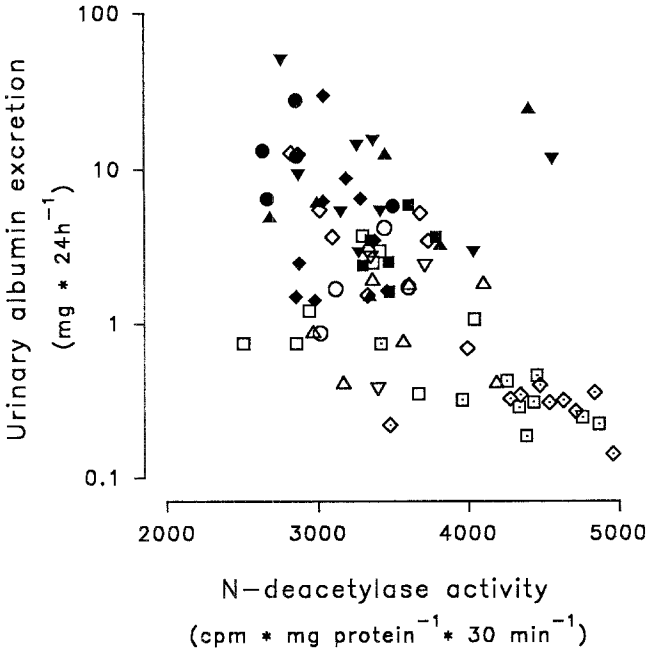

Fig. 3. Glomerular N-deacetylase activity and urinary albumin excretion. Three rat strains were studied (open: H; closed: U; dotted: BB) in various treatment groups (*circle*: C; *square*: D1; *diamond*: D2; *triangle up*: $D1_{\rightarrow 2}$; *triangle down*: $D2_{\rightarrow 1}$). C: Non-diabetic; D1: diabetic, blood glucose 6–10 mmol/l for 3 weeks; D2: diabetic, blood glucose 10–20 mmol/l for 3 weeks; $D1_{\rightarrow 2}$: blood glucose as D1, followed by 2 days as D2; $D2_{\rightarrow 1}$: blood glucose as D2, followed by 2 days as D1

Urinary albumin excretion: Urinary albumin excretion was elevated in the U and low in BB rats ($p < 0.0001$), Table 1. No effect of metabolic control was found in the U or BB, whereas poorly controlled H rats (D2 and $D2_{\rightarrow 1}$) showed a minor increase in albumin excretion ($p = 0.01$).

Glomerular sampling: The number of glomeruli sampled per animal was $29 \pm 6 * 10^3$ (1.02 ± 0.32 mg protein extracted). Kidney weight, and number of glomeruli were slightly lower in U rats ($p < 0.001$) but did not vary between treatment groups (Table 1). No difference in amount of extractable protein per glomeruli were found ($p = 0.45$).

N-deacetylase: Within the diabetic groups significant effects of short- and long-term glucose control on hepatic N-deacetylase activity were found only in the U rats but the tendency was evident also in H rats (Fig. 1). Hepatic N-deacetylase was reduced in U compared to H rats in all groups ($p < 0.001$).

Glomerular N-deacetylase was not significantly linked to short-term glucose control (Fig. 2). However, N- deacetylase activity was reduced in U rats after long-term poor control (U-D2) compared to good control (U-D1) ($p = 0.004$).

Glomerular N-deacetylase activity correlated to urinary albumin excretion (Fig. 3) ($r = -0.60$, $p < 0.0001$). Two U rats (from $UD1_{\rightarrow 2}$ and $U\text{-}D2_{\rightarrow 1}$) diverged markedly from the correlation displayed in Figure 3 (sample volume did not allow reanalysis). Multiple regression analysis (after exclusion of the two outliers), with urinary albumin excretion and blood glucose during the final 24 h before the animal was killed as explanatory variables displayed significant negative correlations between short-term blood glucose control, urinary albumin excretion rate, and glomerular N-deacetylase activity in the U rats (Table 2). In this model, negative correlation coefficients (not significant) for the association between N-deacetylase activity and urinary albumin excretion were also obtained in H and BB rats.

BB rats in poor metabolic control showed a significant reduction in hepatic N-deacetylase activity (Fig. 1). Glomerular N-deacetylase activity was high in BB rats compared to H and U ($p < 0.001$) (Fig. 2). As in the H rats, glomerular N-deacetylase was not affected by metabolic control.

Kidney cortex mRNA: No significant differences in heparan sulphate proteoglycan (Perlecan) or cyclophillin steady-state mRNA levels were found between the treatment groups (Table 3). A reduction in the mean Perlecan/cyclophillin ratio was noted in diabetic animals, but this was not statistically significant.

Table 2. Multiple regression parameters produced with glomerular N-deacetylase activity (dependent variable, log transformed) related to urinary albumin excretion and blood glucose control during the last 24 h

		H	U	BB
Urinary albumin excretion	coefficient	-126 ± 84	-129 ± 62	-55 ± 267
	p-value	0.15	0.046	0.055
Blood glucose (final 24 h)	coefficient	14 ± 14	-32 ± 13	12 ± 18
	p-value	0.34	0.022	0.51
Model	r^2	0.10	0.25	0.23
	p-value	0.27	0.025	0.14

Coefficients are mean ± SEM. Only diabetic animals included

Discussion

The main findings were (i) the effect of short-term blood glucose control on hepatic N-deacetylase activity, (ii) confirmation of increased N-deacetylase vulnerability in the U rats, and (iii) the demonstration of diabetes-induced inhibition of the N-deacetylase in the spontaneously diabetic BB rat. In addition, strain-dependent differences in glomerular N-deacetylase activity was associated with differences in urinary albumin excretion rate.

The mechanism of the N-deacetylase inhibition remains unknown. The effect of short-term metabolic control on hepatic N-deacetylase activity indicates the involvement of a rapid regulatory mechanism, rather than a mechanism related to long-term poor metabolic control, e.g. non-enzymatic glycation. The lack of correlation between hepatic and glomerular N-deacetylase measurements may suggest tissue-specific inhibitory mechanisms, or differentiated triggering of a common mechanism. A 110 kDa glycoprotein component of the N-deacetylase enzyme has been shown to abolish the inhibitive effect of diabetes [6] when added in vitro. Thus, a regulatory mechanism may relate to the biosynthesis or activation of this

Table 3. Kidney cortex mRNA expression of basement membrane heparan sulphate proteoglycan core protein (Perlecan) and cyclophillin

	H					U					BB	
	C	D1	$D1_{\rightarrow 2}$	D2	$D2_{\rightarrow 1}$	C	D1	$D1_{\rightarrow 2}$	D2	$D2_{\rightarrow 1}$	D1	D2
HS	100 ± 21	86 ± 21	84 ± 9	86 ± 31	99 ± 8	74 ± 18	85 ± 32	75 ± 2	94 ± 36	83 ± 14	86 ± 23	94 ± 16
Cyc	100 ± 30	112 ± 33	116 ± 35	96 ± 30	108 ± 11	78 ± 32	94 ± 26	105 ± 27	106 ± 32	111 ± 32	123 ± 36	127 ± 32
HS/Cyc	1.06 ± 0.29	0.80 ± 0.18	0.81 ± 0.36	0.99 ± 0.42	0.92 ± 0.02	1.03 ± 0.27	0.98 ± 0.36	0.82 ± 0.30	0.93 ± 0.35	0.77 ± 0.28	0.79 ± 0.22	0.75 ± 0.14

Expressed as percentage of values in non-diabetic H rats (H-C) (mean ± SD). C, Non-diabetic; D1, diabetic, BG 6–10 mmol/l for 3 weeks; D2, diabetic, BG 10–20 mmol/l for 3 weeks; $D1_{\rightarrow 2}$, BG as D1 followed by 2 days as D2; $D2_{\rightarrow 1}$, BG as D2 followed by 2 days as D1. HS, Heperan sulphate proteoglycan (Perlecan) core protein; Cyc, Cycophillin

component. We measured kidney cortex Perlecan mRNA expression to see if N-deacetylase inhibition was associated with impaired heparan sulphate core protein synthesis. Since only minor diabetes-induced effects on glomerular N-deacetylase were seen, the potential relation to diabetes-induced effects on Perlecan core protein synthesis remains to be resolved. However, our data support previous findings in obese diabetic KK mice showing no significant effect of diabetes on absolute heparan sulphate mRNA expression [24].

The increased hepatic and glomerular N-deacetylase vulnerability in U rats as well as their inherent reduction hepatic N-deacetylase activity, confirms previous findings [5, 7]. In H rats, the absence of a significant effect of blood glucose control on hepatic N-deacetylase activity was associated with a more dispersed distribution of the enzyme activity measurements, presumably reflecting their genetic heterogeneity compared to U rats. Whereas short-term glucose control had no apparent effect on glomerular N-deacetylase activity in any of the strains, long-term poor metabolic control (U-D2) was associated with reduced N-deacetylase activity in the U rats when compared to long-term good control (U-D1).

The urinary albumin excretion rate was measured halfway through the study period to verify the expected grouping into low, medium, and high-level albumin excreting animals (BB, H, and U strain, respectively). A general correlation between urinary albumin excretion and glomerular N-deacetylase activity was found, but no obvious correlation was seen within the strains. We have previously found a negative correlation between the albumin excretion rate and glomerular N-deacetylase activity in long-term (12 weeks) diabetic H but not in U rats [7]. The present study suggests however that an association between glomerular N-deacetylase activity and urinary albumin excretion rate may also exist in the U rat. Thus, control U rats (U-C) which happened to show very high levels of urinary albumin excretion, also had very low glomerular N-deacetylase activity, suggesting an association between constitutional N-deacetylase activity and urinary albumin excretion rate. Therefore, a multiple regression analysis was performed to allow for a combined effect of albumin excretion and blood glucose control. Interestingly, this model displayed significant correlations with N-deacetylase activity of both albumin excretion rate and short-term glucose control in diabetic U rats, but not in H and BB rats. Although no firm conclusions should be drawn from this finding, the results encourage further search for the role of glomerular N-deacetylase activity in the development of increased urinary albumin excretion.

Based on these findings, we suggest that search for a mechanism underlying the diabetes-induced inhibition of the N-deacetylase should address processes influenced by short-term metabolic control. The U rat appears as an attractive model for studies of genetic and tissue specific factors involved in determining the vulnerability of the N-deacetylase.

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