Inhibition of *N*-Acetylheparosan Deacetylase in Diabetic Rats

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The effects on *N*-acetylheparosan deacetylase (*N*-deacetylase) activity exerted by poorly and well-regulated diabetes and variation of genetic background were investigated in insulin-treated streptozocin-induced diabetic rats of two different strains (H and U). *N*-deacetylase plays a key role in heparan sulfate biosynthesis, because *N*-deacetylation is a prerequisite for *N*- and further *O*-sulfation. Specific activity of the enzyme was reduced by 50% in poorly regulated diabetic rats compared with nondiabetic rats ($P <$ 0.001). The decrease in specific activity was accompanied by a reduction in the estimated K_M from 34 ± 3 to 27 ± 4 mg/L ($P < 0.001$). Optimal insulin treatment, leading to near normalization of blood glucose, prevented reduction in *N*-deacetylase activity. In rat strain U, however, a 20% reduction was found despite optimal insulin treatment ($P = 0.01$), and the nondiabetic animals of this strain had reduced *N*-deacetylase activity compared with nondiabetic rats from the H strain. This might suggest a genetic difference between the rat strains in the regulation of the enzyme activity. The diabetes-induced inhibition of *N*-deacetylase may have an important role in the pathogenesis of nephropathy and vascular complications in human diabetes mellitus. *Diabetes* 40:1449–52, 1991

Diabetes-induced alterations of cellular and basement membrane heparan sulfate may represent an important pathogenetic mechanism in the development of late diabetic vascular complications. Several studies have confirmed that experimental diabetes impairs heparan sulfate synthesis (1–4) and decreases the degree of heparan sulfate sulfation (5–8). Reduced concentration and reduced sulfation of heparan sulfate may lead to increased glomerular permeability due to loss of glomerular basement membrane–fixed negative charge (9). Also, the important anticoagulant function of the endothelial heparan sulfate depends on the degree of heparan sulfate sulfation (10–12); therefore, reduced heparan sulfate sulfation could possibly lead to acceleration of atherogenesis. Both loss of glomerular charge selectivity and increased atherogenesis characterize the severe angiopathy in patients developing diabetic nephropathy (13). Moreover, clinically apparent intraindividual differences in the development of severe diabetic angiopathy may be related to the enzymes involved in the metabolism of heparan sulfate (13). Therefore, it is of considerable clinical interest to study enzymes involved in the biosynthesis of heparan sulfate.

The enzyme *N*-acetylheparosan deacetylase (*N*-deacetylase) plays a key role in the biosynthesis of heparan sulfate, because *N*-deacetylation of the heparan sulfate glucosamine units is prerequisite to *N*-sulfation and further modifications of the polymer (14–16). Inhibition of *N*-deacetylase activity will therefore impair sulfation of heparan sulfate. Because sulfation of heparan sulfate is impaired in diabetic animals, we studied the influence of diabetes on *N*-deacetylase activity. Furthermore, because intraindividual differences in susceptibility for diabetic complications may be related to enzymes such as *N*-deacetylase, we studied two genetically different Sprague-Dawley rat strains, denoted H and U. U rats are characterized by diabetes-induced alterations in the synthesis of extracellular matrix components and a high rate of skeletal malformations among the offspring (17–20).

RESEARCH DESIGN AND METHODS

Diabetes was induced in adult H and U Sprague-Dawley rats with an injection of streptozocin (55 mg/kg i.v. in 0.05 M citrate buffer, pH 4.2; S-0130, Sigma, St. Louis, MO). All rats were insulin treated (heat-treated, Ultralente beef, Novo, Bagsvaerd, Denmark) and randomized to either good (D1; blood glucose 6–10 mM) or poor (D2; blood glucose 15–20

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Received for publication 11 January 1991 and accepted in revised form 15 May 1991.

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mM) metabolic control. Insulin was given subcutaneously and in the afternoon to obtain optimal effect (21). Morning blood glucose was measured daily (Hypocount; BM-Test-BG Stix; Ercopharm, Vedback, Denmark), and urine was tested for ketone bodies by dipstick. Morning blood glucose represented the nadir of 24-h blood glucose (21). Nondiabetic rats from each strain were used as controls. Animals were killed by decapitation after 8–10 wk. All rats had free access to water and standard fodder.

The ventromedial liver lobe was removed rapidly and homogenized (Polytron, 4 min on ice) in 10-fold vol lysate buffer (0.05 M Tris, 1% Triton X-100, and 2 mM EDTA, pH 7.5). The time from the killing of the rat until the end of the homogenization was <6 min. The homogenized sample was left on ice for 1 h, after which the microsomal fraction was isolated as the supernatant by centrifugation for 5 min at 12,000 × *g*. Samples were stored at −80°C.

The *N*-deacetylase assay followed essentially the method of Navia et al. (22) with *N*-[^{3}H]acetyl–labeled *Escherichia coli* K5 capsular polysaccharide as substrate. The polysaccharide was donated by G. van Deedem (Diosynth, Netherlands). This material was purified further on an ion-exchange column (DEAE) with a salt-gradient elution (0.05–1.50 M NaCl) at pH 4. ^{3}H-labeled acetyl was incorporated as *N*-acetyl groups as described earlier (22). The size of the labeled substrate was identical (50,000–60,000 M_r) to the unlabeled substrate (verified by chromatography on Superose gel high-performance liquid chromatography column [Pharmacia, Uppsala, Sweden]). Labeled substrate was mixed with unlabeled substrate until the specific activity was 2×10^6 cpm/mg polysaccharide. Liver microsomal fraction (0.5–1.0 mg protein) was incubated with substrate in 50 mM 2-[*N*-morpholino]-ethanesulphonic acid and 10 mM $MnCl_2$ (pH 6.3) in a total volume of 200 μl. After 30 min at 37°C, the reaction was terminated by addition of 200 μl stopping solution (1 M monochloroacetic acid, 0.5 M NaOH, and 2 M NaCl). [^{3}H]acetate release was measured by scintillation counting in a biphasic system with 5 ml scintillation fluid (Optiscint "Safe" [Pharmacia] with 10% isoamyl alcohol) added to the assay mixture. Blank samples, where the stopping solution was added before the substrate, were included in each assay.

N-deactylase activity was measured in duplicate at seven substrate concentration levels from 9 to 600 mg/L in each sample. The sample-blank ratio (cpm) was 2:1 at the highest substrate concentration and 6:1 at the lowest. Inter- and intraassay coefficients of variation were 10 and 3%, respectively. The samples were randomly distributed in the 16 assays performed, and the technician was unaware of their treatment group. *N*-deacetylase specific activity at saturating substrate concentration (cpm × s^{-1}) expressed per gram total protein and the K_M were estimated from the Eadie-Hofstee plot (23). The linearity in the Eadie-Hofstee plots was good ($r = 0.987$). However, the point at the highest substrate concentration (600 mg/L, 20-fold of estimated K_M) tended to diverge from the regression line. This was possibly related to the relatively poor sample-blind ratio (cpm) of 2:1 at that point. Thus, this point was subsequently removed from all regressions, which lead to a general reduction in the estimated enzyme activity but otherwise did not influence the significance of the results.

Total protein was measured by a Bradford protein assay purchased from Bio-Rad (Richmond, CA) with albumin as the standard. Other measurements, performed on a Cobas Mira automatic analyzer, were of glucose, creatinine, aspartate aminotransferase (ASAT), β-hydroxybutyate, and triglyceride.

Two-tailed nonparametric statistics (Mann-Whitney *U* test and Spearman rank correlation coefficient) were used.

RESULTS

Mean ±SD blood glucose was 7.0 ± 0.8 mM in D1 groups and 17.3 ± 0.9 mM in D2 groups without difference between H and U rats. More parameters of metabolic control are given in Table 1. Generally, poor metabolic control in D2 resulted in increased levels of serum β-hydroxybutyrate and triglyceride, which were normal in D1. The status of the hepatic metabolism was further evaluated by measuring hepatocyte ASAT activity. This enzyme participates in the oxidative de-

TABLE 1
Parameters related to metabolic control

	Rat strain H			Rat strain U		
	Control	Group D1	Group D2	Control	Group D1	Group D2
n (M/F)	6/4	4/2	3/2	6/4	6/3	4/3
Weight (g)						
Male	515 (500–535)	475 (455–500)	420 (395–425)	470 (405–530)	505 (420–555)	450 (430–470)
Female	295 (280–325)	305 (300–310)	230 (205–255)	280 (260–310)	305 (325–295)	250 (240–275)
Blood glucose (mM)*	3.8 (3.3–4.6)	6.8 (6.7–7.7)	16.8 (16.5–17.5)	3.4 (2.1–4.0)	6.8 (5.4–8.5)	17.5 (16.3–19.4)
Insulin dose (U/kg)		15.5 (13.0–18.4)	4.3 (3.2–5.0)		12.1 (10.5–16)	3.8 (2.6–6.0)
Serum ketones (mM)†	0.5 (0.4–0.7)	0.5 (0.4–1.7)	1.3 (0.7–2.1)	0.6 (0.3–0.9)	0.6 (0.3–0.8)	0.7 (0.6–0.7)
Serum triglyceride (mM)	1.7 (0.8–2.2)	1.2 (0.9–1.7)	3.0 (2.3–5.9)	1.6 (1.1–2.8)	1.3 (0.7–2.3)	2.5 (0.8–3.1)
Hepatic aspartate amino transferase (U/g protein)	2.2 (1.6–2.6)	2.7 (2.1–2.8)	3.7 (3.4–4.0)	2.8 (2.2–3.1)	2.8 (2.2–3.6)	3.9 (2.6–4.5)

Nondiabetic rats were used as controls. D1, diabetic in optimal metabolic control; D2, diabetic in poor metabolic control. Values are medians with ranges in parentheses.
*From last 2 wk before killing.
†β-Hydroxybutyrate.

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generation of amino acids, and the activity was significantly elevated in the poorly regulated animals but normal in D1.

Hepatic *N*-deacetylase specific activity (cpm · s^{-1} · g^{-1} total protein) was reduced by 50% in both H and U rats (mean ± SD 364 ± 67 and 387 ± 84, respectively) with poor metabolic control compared with nondiabetic rats (785 ± 42 and 710 ± 122, respectively) (Fig. 1). The more intensive insulin treatment in D1 restored *N*-deacetylase activity, but a significant reduction (20%) was still present in U rats (H-D1, 687 ± 135, $P = 0.06$ vs. H controls; U-D1, 581 ± 90, $P = 0.01$ vs. U controls). Reduction in mean ± SD *N*-deacetylase activity was accompanied by a reduction in K_M from 34 ± 3 in controls to 27 ± 4 mg/L in D2 ($P < 0.001$). There was a positive correlation between enzyme activity and K_M ($r = 0.68$, $P < 0.001$; Fig. 2). Within the treatment groups, this correlation was significant in D1 ($P = 0.01$) but not in controls ($P = 0.11$) or D2 ($P = 0.18$), although the slopes of the best-fit regression lines from each group appeared quite similar. There was no difference in mean ± SD liver size (male, 16.5 ± 3; female, 9.3 ± 1 g) or macroscopic appearance between H and U rats or between the treatment groups.

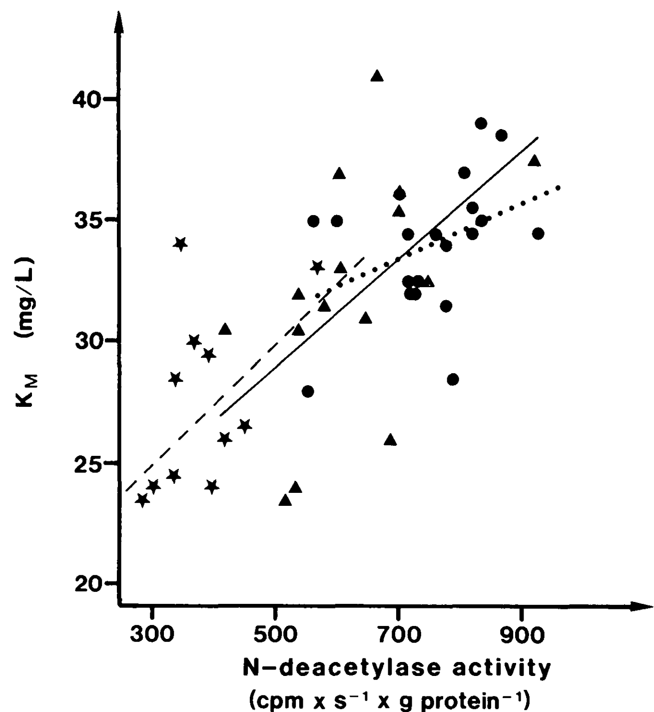

FIG. 2. ***N*-acetyl-heparosan-deacetylase activity plotted against K_M. Three groups are indicated (with regression lines in parentheses): ●; nondiabetic rats (*dotted line*); ▲, diabetic rats in optimal metabolic control (*solid line*); ⋆, diabetic rats in poor metabolic control (*dashed line*). $r = 0.68$, $P < 0.001$.**

DISCUSSION

The heparan sulfate polysaccharide chains are initially composed of D-glucuronic acid (GlcA) and *N*-acetyl-D-glucosamine (GlcNAc) units in a $(GlcA\text{-}GlcNAc)_n$ structure. Subsequent modification of the polysaccharide is initiated by *N*-deacetylation and *N*-sulfation. Other modifications are then C5 epimerization of glucuronic acid and *O*-sulfation in various positions, both of which appear to occur exclusively in the vicinity of previously incorporated *N*-sulfate groups (for review, see refs. 14–16). The initial step, i.e., *N*-deacetylation, is catalyzed by *N*-deacetylase, and inhibition of this enzyme will therefore impair the sulfation of heparan sulfate.

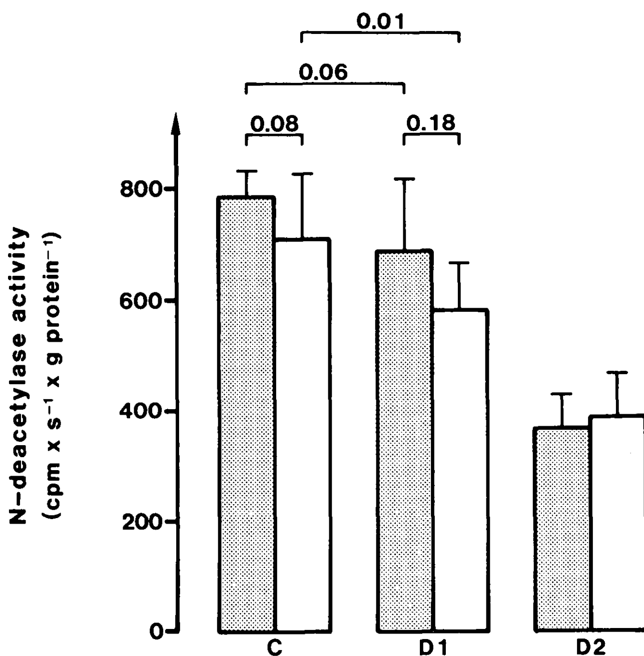

FIG. 1. **Mean ± SD of *N*-acetyl heparosan deacetylase activity. Effect of diabetes was studied in 2 rat strains, H (*shaded bars*) and U (*open bars*). *C*, nondiabetic controls; *D1*, diabetic in optimal metabolic control; *D2*, diabetic in poor metabolic control. *P* values (nonparametric, unpaired 2 tailed) indicated on figure. Differences between *D1* and *D2* were significant ($P < 0.01$) in both strains.**

Our study demonstrates that *N*-deacetylase is significantly inhibited in streptozocin (STZ) induced diabetic rats and that insulin treatment reverses the inhibition. Thus, diabetes inhibits the activity of a key enzyme in heparan sulfate biosynthesis. Moreover, it was found that, in the U rat strains, *N*-deacetylase activity could not be normalized despite excellent metabolic control completely restoring serological and hepatic measurable signs of metabolic disorder. It is also possible that the *N*-deacetylase activity was not completely normalized in the H-D1 rats and that the statistical insignificance was due to the smaller number of rats in this group. Although there was no firm evidence of a difference in *N*-deacetylase activity between diabetic U and H rats, note that mean *N*-deacetylase activity appeared to be reduced in the U rats in control and D1 compared with H rats (Fig. 1), because previous studies have demonstrated strain differences in the biosynthesis of chondroitin sulfate proteoglycans in rib cartilage (U values lower than H values; 20). We subsequently measured hepatic *N*-deacetylase activity in a small follow-up study in nondiabetic H and U rats and again found that U rats ($n = 10$) had lower mean *N*-deacetylase activity (459 [range 444–565] cpm × s^{-1} × g^{-1} protein) compared with H rats ($n = 9$; 494 [463–526] cpm · s^{-1} · g^{-1} protein, $P = 0.008$). Together, the findings might suggest a genetic difference between the rat strains in the regulation of *N*-deacetylase activity.

Although some studies indicate that diabetes does not impair heparan sulfate sulfation (1,2), others have demon-

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strated reduction in heparan sulfate sulfation (5–8). This study supports the latter finding. Hepatic N-deacetylase was measured because the liver offers access to amounts of relatively homogenous tissue needed for the enzyme analysis and because diabetes-induced reduction in sulfation of hepatic heparan sulfate has been demonstrated previously (6). It is possible that N-deacetylase inhibition is specific for hepatic tissue, e.g., secondary to diabetes-induced regulation of general hepatic metabolism, and that the inhibition has no relevance for heparan sulfate metabolism in organs severely affected by diabetic vasculopathy, such as the kidney and retina. That U rats had reduced N-deacetylase activity, despite complete normalization of hepatic ASAT, speaks against this, however. One recent study found that the hepatic activity of another heparan sulfate polymer modifying enzyme (epimerase) was unaltered despite a diabetes-induced 50% reduction in N-deacetylase activity (E. Unger, Biomedicum, Uppsala, Sweden, unpublished observations). A definite answer to whether inhibition of N-deacetylase is specifically hepatic must await studies of N-deacetylase activity from other tissue sources.

We measured the N-deacetylase activity at saturating substrate concentration, but because we have not measured the absolute enzyme concentration ([E]), it is not possible to tell whether the reduced activity reflects changes in [E] or a reduction in the catalytic rate constant (k_{cat}) of the overall enzyme action. However, the significant reduction in K_M in D2 strongly indicates the presence of noncompetitive inhibitor of N-deacetylase activity if analyzed in terms of the Michaelis-Menten formalism and under the assumption of reversible inhibition. Noncompetitive inhibition is characterized by combined reduction in V_{MAX} (i.e., k_{cat}, where $k_{cat} = V_{MAX}/[E]_0$) and K_M. Although the presence of a noncompetitive inhibitor is demonstrated most clearly in group D2, the significant positive correlation between the specific enzyme activity and K_M and the similarity between the best-fit regression lines of D1 and D2 (Fig. 2) suggest that this type of inhibition might be acting also at more optimal levels of blood glucose control. The exact mechanism of N-deacetylase action is unknown, but it has been demonstrated that subsequent sulfation stimulates N-deacetylase activity (24). Therefore, it is possible that reduction in N-deacetylase activity is combined with a reduction in postdeacetylation sulfation capacity, which in turn might cause a nonproductive binding of the substrate to the N-deacetylase, resulting in noncompetitive inhibition of this enzyme.

We conclude that N-deacetylase activity is inhibited by diabetes. Furthermore, we found evidence to suggest strain differences in the activity of this enzyme. Inhibition of a key enzyme in heparan sulfate polymer modification could cause a reduction in heparan sulfate sulfation and thereby play an important pathogenetic role in the development of diabetic vascular complications. It needs to be established whether the inhibition of N-deacetylase is present not only in liver cells but also in the glomerular epithelium and vascular endothelium, the cells directly involved in development of diabetic angiopathy.

ACKNOWLEDGMENTS

This study was supported by a grant from the Danish Diabetes Association, Swedish Medical Research Council Grant 12X-7475, and The Nordisk Insulin Foundation Committee.

We acknowledge the invaluable assistance of Lena Kjellén in establishing the N-deacetylase assay in our laboratory. We are grateful for fruitful discussions with Dr. Torsten Deckert and Assistant Professor Carl J. Hedeskov during the course of this study. Karin Jensen is thanked for superb technical assistance.

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