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Glucosaminyl N-deacetylase in cultured fibroblasts: comparison of patients with and without diabetic nephropathy, and identification of a possible mechanism for diabetes-induced N-deacetylase inhibition

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Summary. Impaired heparan sulphate biosynthesis through diabetes-induced inhibition of glucosaminyl N-deacetylase may have a central role in the development of diabetic nephropathy, and genetic differences in the vulnerability of the N-deacetylase could influence the risk of developing nephropathy. We studied N-deacetylase activity in fibroblast cultures from Type 1 (insulin-dependent) diabetic patients with (n = 14) or without (n = 13) diabetic nephropathy, together with non-diabetic control subjects (n = 7). No difference in N-deacetylase activity was found (p = 0.13), and no inhibition of N-deacetylase was found in cultures grown at 25 mmol/l glucose. N-deacetylase activity was inversely correlated to growth rate (r = –0.59, p = 0.0008), and in patients with nephropathy a negative correlation between HbA_{1c} and fibroblast N-deacetylase activity (r = –0.72, p = 0.012) was found. Cell-cycle analysis revealed an increased fraction of S-phase cells in patients with nephropathy (28 % (21–52 %)) compared to healthy control subjects (17 % (9–24 %)), p = 0.0008, but not between patients with and without nephropathy (latter group 26 % (11–43 %)), p = 0.43. Forskolin, an activator of protein kinase A, specifically decreased N-deacetylase activity, whereas activation of protein kinase C produced a combined reduction in N-deacetylase activity and total protein synthesis. In conclusion, no constitutive defects in N-deacetylase activity were found in fibroblasts from these patients. Further studies should consider possible associations between fibroblast characteristics and pre-biopsy environmental parameters related to cellular memory phenomena. Finally, activation of protein kinase A provides a potential general pathway for regulating N-deacetylase activity.

Key words: Diabetic nephropathy, heparan sulphate, genetic predisposition, fibroblast, cell culture, growth rate, cell-cycle, protein kinase A, protein kinase C.

A genetic factor seems to influence the individual risk of developing diabetic nephropathy in Type 1 (insulin-dependent) diabetic patients [1]. Identification of this factor is however hampered by our current lack of knowledge concerning the pathogenesis of diabetic nephropathy.

A potentially central pathogenetic element is the impairment of heparan sulphate proteoglycan metabolism [2–5]. As a widespread extracellular component, the functional roles of heparan sulphate include maintenance of glomerular charge selectivity [6, 7], endothelial anticoagulant function and lipid metabolism [8], and regulation of cellular growth [9]. Reduced sulphation of the heparan sulphate glycosaminoglycan chains, which may lead to impairment of any of these functions, has been demonstrated in experimental diabetes [10, 11]. The sulphation of heparan sulphate takes place in the Golgi apparatus, initially under the control of the enzyme glucosaminyl N-deacetylase (EC no. 3.5.1.33) [12]. The activity of this enzyme is markedly inhibited in experimental diabetes [13, 14]. Furthermore, evidence of genetically determined differences in the vulnerability of the N-deacetylase has been found in closely-related rat strains [15]. This combination of (i) key role in heparan sulphate synthesis, (ii) diabetes-induced inhibition, and (iii) genetically-determined vulnerability, indicates that N-deacetylase is a major candidate to be a genetically controlled risk factor.

The impact of genetic factors on cellular biology may be studied using individual cell cultures. Thus, skin fibroblast cultures from patients with diabetic nephropathy have recently been found to express abnormalities in heparan sulphate biosynthesis as well as in regulation of growth and intracellular pH [16–18]. We have therefore studied N-deacetylase activity in skin fibroblast cultures from diabetic patients, hypothesizing that reduced activity, or related abnormalities in the regulation of the activity when exposed to diabetes-associated environmental factors, would be found in cultures from patients with diabetic nephropathy. The fibroblast cultures were exposed to high or

Table 1. Study population

	Control subjects	D1	D2
n/(male)	7/(5)	13/(7)	14/(8)
Age (years)	33 (28–34)	40 (29–48)	39 (32–53)
Diabetes duration (years)	–	25 (12–44)	25 (16–38)
HbA_{1c} (%)	–	8.4 (6.3–10)	9.2 (8.2–11.1)
Anti-hypertensive treatment (*n*)	0	0	14
Retinopathy (None/background/proliferative)	–	6/6/1	0/2/12

Data are median (range) or counts. D1, Diabetic patients with normal urinary albumin excretion; D2, diabetic patients with overt diabetic nephropathy

Table 2. Cell growth rate as determined by cell count and by cell-cycle analysis

	Control subjects	D1	D2	*p*-value[a]
Growth rate[b] (% per day)	22 (8–50)	33 (11–51)	24 (–7–51)	0.56
G1-phase[c] (%)	68 (58–79)	59 (34–79)	52 (41–71)	0.039
S-phase[c] (%)	17 (9–24)	26 (11–43)	28 (21–52)	0.007
G2 + M-phase[c] (%)	17 (13–19)	16 (8–23)	14 (6–24)	0.82

[a] By Kruskall Wallis test. Between the groups, significant differences (Mann Whitney test) in G1- and S-phase fractions were found between C and D2 ($p = 0.006$ and $p = 0.0008$, G1- and S-phase, respectively), but not between C and D1 ($p = 0.20$ and $p = 0.09$) or between D1 and D2 ($p = 0.50$ and $p = 0.43$). [b] Based on cell counting 3 days after plating. [c] Flow cytometric analysis of DNA content was performed as described in Subjects, materials and methods, and the fraction of cells in G1, S, and M + G2-phase calculated. D1, Diabetic patients with normal urinary albumin excretion; D2, diabetic patients with overt diabetic nephropathy

to normal glucose concentration. In addition, since activation of protein kinase A or C may be partly responsible for the abnormal basement membrane metabolism in diabetic angiopathy [19], the effect of protein kinase A or C activation on N-deacetylase activity was tested.

Subjects, materials and methods

Study population: Twenty-seven Type 1 diabetic patients and seven healthy subjects participated in the study. The diabetic patients had either normal urinary albumin excretion (less than 30 mg/24 h, $n = 13$, group D1) or overt diabetic nephropathy (albumin excretion above 300 mg/24 h without other known cause than diabetic nephropathy, $n = 14$, group D2). Patients in group D2 had a median serum creatinine of 99 μmol/l (range 77 to 135), and had developed persistent proteinuria within 15 (11 to 32) years of diabetes duration. Further clinical characteristics are given in Table 1. The study was approved by the local ethical committee and informed consent obtained from all participants.

Culture conditions: Primary skin fibroblast cultures were established from skin punch biopsies (4 × 4 mm) taken from the deltoid region. Cells were grown in Minimal Essential Medium-Eagle (all reagents purchased from Gibco, Roskilde, Denmark, unless otherwise stated) with 15% fetal calf serum, 2 mmol/l L-glutamine, 200 IU/ml penicillin and 200 μg/l streptomycin. All cultures were grown at 5 mmol/l glucose, the medium was changed three times per week, and cultures were split (ratio 1:2) when confluent. The experiments were performed after 6 to 10 passages. Seven days before the final harvest, parallel cultures were grown (split 1:2 once after 4 days) in medium supplemented with 25 mmol/l or 5 mmol/l glucose. Culturing and experiments were performed by a technician who was unaware of the patients identity.

Growth rate: Individual growth rates were measured by cell counting and by cell-cycle analysis. Cells ($2 \cdot 10^5$) from confluent cultures, were plated in a 80 cm^2 culture flask with 20 ml medium. After 72 h the cells were harvested, counted under the microscope using a Bürker-Türck counting chamber, and stored at –20 °C until cell-cycle analysis was performed. After thawing, cells were stained with 500 μl of lysis-DNA-solution [20] consisting of calcium and magnesium free Dulbecco's phosphate buffered saline (0.2 g/l KCl, 0.2 g/l, KH_2PO_4, 8 g/l NaCl, 2.16 g/l $Na_2HPO_4 \cdot 7H_2O$), 0.5% volume/volume Nonidet P-40, 20 μg/ml propidium iodide (Sigma, St. Louis, Mo., USA) 0.2 mg/ml RNase (R-5503, Sigma), and 0.5 mmol/l EDTA at pH 7.2 for 30 min at 4 °C. The propidium iodide fluorescence (linear scale) was then measured in a FACScan flow cytometer (Becton Dickinson, Glostrup, Denmark), with a flow rate of 100–500 counts per second, using the program Cell Fit, sum of broadened rectangles.

N-deacetylase measurements: Confluent cultures were washed twice in Hanks' balanced salt solution (HBSS) (0.4 g/l KCl, 0.06 g/l KH_2PO_4, 8 g/l NaCl, 0.09 g/l $Na_2HPO_4 \cdot 7H_2O$, 1 g/l D-glucose) and harvested by scraping. The cell pellet was suspended in 300 μl hypotonic detergent buffer (0.05 mol/l Tris, 1% Triton-X-100, 2 mmol/l EDTA) for 10 min at room temperature. Protein concentration in the microsomal fraction (i.e. the supernatant after centrifugation at 12,000 *g* for 2 min) was assayed with the bicinchoninic acid protein assay reagent (Pierce, Rockford, Ill., USA) on a Cobas Mira (Roche, Hvidovre, Denmark) automated analyser, using human reference serum (Orion Diagnostics, Espoo, Finland) as the standard. Total microsomal protein (25 μg) was used for N-deacetylase activity measurement, according to the method of Navia et al. [21], with a 50,000 kDa N-[^{3}H]-acetyl-labelled *Escherichia coli* K5 capsular polysaccheride (5 mg/l) as the substrate [14]. The assay was performed as previously described [15]. Within and between assay coefficients of variation were below 5% and 10% respectively. Results are expressed as cpm · 25 μg total $protein^{-1} \cdot 30\ min^{-1}$.

Effect of protein kinase A or C activation on N-deacetylase activity: Confluent cultures from healthy control subjects were incubated for 18 h with 100 μmol/l forskolin (Sigma, F-6886) or 0.08 to 50 μg/l phorbol-12,13-dibutyrate (PDBU) (Sigma, P-1269). The effect of short-term PDBU (50 μg/l) incubation on N-deacetylase activity was measured after 0.5, 1, 2, 4, 8, and 60 min. Forskolin stimulates adenylatcyclase leading to increased cAMP formation and protein kinase A activation, while PDBU activates protein kinase C by mimicking the effect of diacylglycerol.

Total protein synthesis: To evaluate the specificity of the effect of protein kinase A or C activation on N-deacetylase activity, the effect of forskolin or PDBU on total protein synthesis rate was measured. Cultures in 7 cm^2 dishes were incubated with 1 μCi ^{3}H-labelled leucine (L-[4, 5-^{3}H]-leucine; Amersham Int., Amersham, Bucks., UK) in leucine-free Eagles medium MEM with 1% fetal calf serum, following 18 h of incubation with forskolin or PDBU as described above. After 1 h, cells were washed four times with ice-cold HBSS and subsequently lysed by adding 1 ml 0.1% SDS and leaving them to stand for 30 min at 37 °C. Protein was precipitated by adding 0.1 ml 100% trichloroacetic acid and the sample left at 4 °C for 4 h. The precipitated protein was captured on a BetaPlate filter mate A (Pharmacia, Uppsala, Sweden) using a suction device (Skatron, Lier, Norway), washed twice in 2.5 ml 4% trichloroacetic acid, and ^{3}H incorporation counted in a LKB BetaPlate scintillation counter.

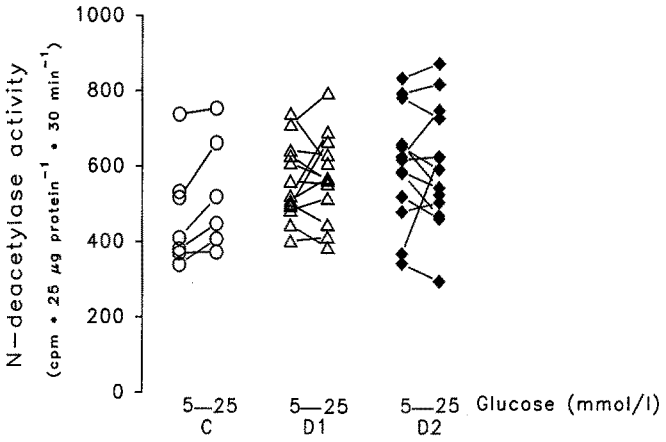

Fig. 1. N-deacetylase activity in primary skin fibroblast cultures from healthy control subjects (C), diabetic patients with normal urinary albumin excretion (D1), and diabetic patients with overt diabetic nephropathy (D2). Cells were cultured under normal (5 mmol/l) and high (25 mmol/l) glucose conditions. High glucose conditions induced a significant increase in N-deacetylase activity in healthy control subjects ($p = 0.04$). The groups were otherwise not significantly different ($p = 0.13$)

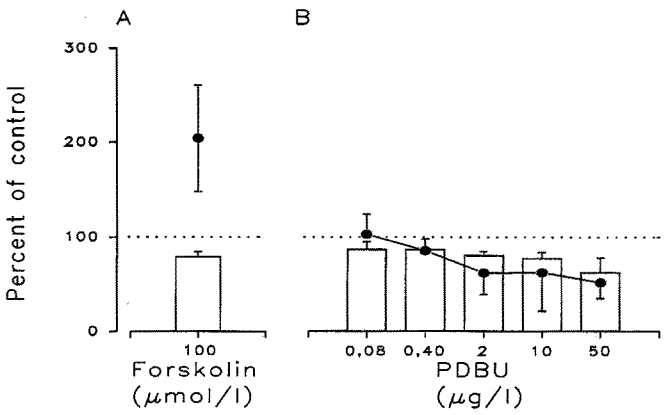

Fig. 3 A,B. Effect on N-deacetylase activity (bars) and total protein synthesis (•) of **A**: protein kinase A activation (by 18 h incubation with 100 µmol/l forskolin) and **B**: protein kinase C activation (by 18 h incubation with 0.08 to 50 µg/l phorbol-12,13-dibutyrate (PDBU)) in primary skin fibroblast cultures from healthy control subjects ($n = 4$). Results are expressed as percent of individual parallel control cultures. Error bars indicate 95% confidence intervals

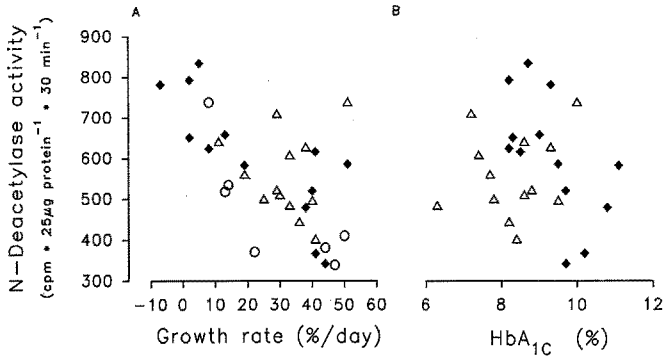

Fig. 2 A,B. Correlations between N-deacetylase activity, cell growth rate, and HbA_{1C} in primary skin fibroblast cultures from healthy control subjects (○), diabetic patients with normal urinary albumin excretion (△), and diabetic patients with overt diabetic nephropathy (◆). **A**: Cell growth rate was calculated from cell counting following 72 h growth of $2 \cdot 10^5$ skin fibroblasts plated in a 7 cm^2 culture dish. A negative correlation between cell growth rate and N-deacetylase activity was found ($r = -0.59$, $p = 0.0008$). **B**: HbA_{1C} was measured in the diabetic patients at the time of the skin biopsy, after which cells were cultured for 2 to 3 months under standardised in vitro conditions. A negative correlation between HbA_{1C} and N-deacetylase activity was found in patients with overt nephropathy ($r = -0.72$, $p = 0.01$) but not in patients with normal albumin excretion (r = 0.19, $p = 0.51$)

Statistical analysis

Non-parametric tests (Wilcoxon's signed rank sum test, Mann-Whitney rank sum test, Kruskall Wallis one-way analysis by rank, and Spearman rank correlation) were used. Two-tailed p-values below 0.05 were considered significant. Results are presented as median(range) unless otherwise stated.

Results

N-deacetylase activity in the skin fibroblast cultures grown at 5 mmol/l glucose did not differ between the groups ($p = 0.13$), and no general effect on N-deacetylase activity was found after culturing at 25 mmol/l glucose for 7 days (Fig. 1). In healthy control subjects however, cultures grown at 25 mmol/l glucose showed a minor increase in N-deacetylase activity from 410(339–737) to 484(372–753), $p = 0.04$ (Fig. 1).

There was no difference between the groups in growth rate as determined by cell counting (Table 2). The cultures reached 25% (7–77%) of their confluent density during the 72 h of the growth rate experiment (C: 23% (14–38%); D1: 45% (8–77%); D2: 25% (7–68%)). In contrast, the fraction of cells in the S-phase after 72 h was significantly increased in group D2 compared to group C ($p = 0.0008$), whereas group D1 did not differ from either C ($p = 0.09$) or D2 ($p = 0.43$), Table 2.

Similar results were obtained, when only D1 patients with a diabetes duration above 25 years ($n = 7$) and group D2 patients with onset of diabetic nephropathy before 15 years of diabetes duration ($n = 8$) were included in the analysis, (not shown).

N-deacetylase activity in cultures grown at 5 mmol/l glucose correlated positively to age (r = 0.43, $p = 0.016$) and negatively to growth rate ($r = -0.59$, $p = 0.0008$) (Fig. 2a). Interestingly, there was a relatively strong negative correlation between HbA_{1C} and N-deacetylase activity in patients from D2 ($r = -0.72$, $p = 0.012$), but no such correlation within group D1 (r = 0.19, $p = 0.51$) (Fig. 2b). Similar results were obtained in cultures grown at 25 mmol/l glucose (data not shown).

Overnight incubation with forskolin induced a 25% reduction in N-deacetylase activity while increasing total protein synthesis rate almost two-fold (Fig. 3a). The amount of extractable protein per culture was not significantly increased by forskolin (mean ± SD: 108 ± 8% of control). PDBU induced a reduction both in N-deacetylase activity and in total protein synthesis rate (Fig. 3b). No effect of short-term PDBU (50 µg/l) incubation on N-deacetylase activity was found (data not shown).

Discussion

The study failed to demonstrate an inherited reduction in N-deacetylase activity in skin fibroblast cultures from patients with diabetic nephropathy. High glucose had no general effect on N-deacetylase activity, whereas stimulation of protein kinase A activity caused a specific reduction in N-deacetylase activity. This provides new insight into the mechanism of diabetes-induced N-deacetylase inhibition, as well as information which can be used to determine the role of abnormal heparan sulphate metabolism in the development of diabetic nephropathy.

The lack of difference in N-deacetylase activity between the groups suggests that no major genetic defect leading to chronic widespread N-deacetylase malfunction is present in patients developing diabetic nephropathy. Since high glucose failed to produce the diabetes-induced inhibition of N-deacetylase activity previously found in vivo, the present study conditions may not expose potential differences in the vulnerability of the N-deacetylase. Thus we cannot exclude a role for N-deacetylase in the genetic predisposition to diabetic nephropathy. Two findings, (i) the effect of glucose on N-deacetylase activity in control subjects and (ii) the correlation between N-deacetylase activity and HbA_{1c} in patients from group D2, seems to challenge the fundamental presumption that skin fibroblast cultures grown in vitro for 2 to 3 months are devoid of previous in vivo environmental factors, e.g. diabetic metabolic control. Indeed, a memory phenomenon associated with high glucose in vitro has previously been described [22]. If skin fibroblasts from diabetic patients have been permanently marked by the antecedent in vivo metabolic state, the correlation between N-deacetylase activity and HbA_{1c} in patients with diabetic nephropathy may indicate the presence of a regulatory response not found in patients without diabetic nephropathy. Further experimental support for this notion must be found before drawing any conclusions.

Increased intracellular pH, Na^+/H^+ antiport activity, and thymidine incorporation as recently reported suggests that skin fibroblast from patients with diabetic nephropathy may be characterized by an increased rate of proliferation [17, 18]. This is of potential interest in relation to heparan sulphate metabolism. In agreement with the known growth-regulatory effects of heparan sulphate [9, 23], we found an inverse correlation between growth rate and N-deacetylase activity. There was however no difference in growth rate, as calculated from cell counting, between our study groups. The cell-cycle analysis did indicate increased proliferative activity (at the time of harvest) in cultures from patients with nephropathy compared to healthy control subjects as also suggested by Trevisan et al. [18], but no difference was found between our diabetic patients with and without nephropathy. Thus, during standard culture conditions, no difference in the rate of proliferation between fibroblasts from patients with and without diabetic nephropathy was found. Under these conditions an increased rate of entrance into the S-phase may be linked to the diabetic state per se, perhaps related to a cellular memory phenomenon as discussed above. Clearly, the methodological differences between studies may expose different biological aspects, and future studies should continue to address abnormalities in growth rate related parameters between the patient groups.

Incubation with forskolin specifically reduced N-deacetylase activity. We have previously found very rapid and complementary effects of insulin dose shifts on hepatic N-deacetylase activity in streptozotocin-diabetic rats [24]. Insulin inhibits protein kinase A activity by decreasing the binding of cAMP to the enzyme [25, 26]. Our study may therefore have identified a general pathway, i.e. protein kinase A activity, for regulation of N-deacetylase activity. Insulin resistance may be associated with a decrease in insulin-induced protein kinase A down-regulation [26]. This could provide an explanation for the postulated association between development of diabetic nephropathy [27] and the presence of insulin resistance, involving decreased N-deacetylase activity. The effect of protein kinase C activation as induced by PDBU on N-deacetylase activity in our study seems related to a general toxic rather than a specific effect since total protein synthesis was down-regulated in parallel.

We conclude that whereas a role for N-deacetylase in the development and genetic susceptibility to diabetic nephropathy may not be excluded by the present study, no major constitutive defects in N-deacetylase activity is found in skin fibroblast cultures from patients with diabetic nephropathy. Further studies should consider possible associations between skin fibroblast culture characteristics and pre-biopsy clinical parameters such as HbA_{1c}-related cellular memory phenomena. Finally, activation of protein kinase A provides a potential general pathway for regulation of N-deacetylase activity.

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