Na^+/H^+ Exchanger Isoform-1 Abundance in Skin Fibroblasts of Type I Diabetic Patients With Nephropathy

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In diabetic nephropathy and essential hypertension, the cellular Na^+/H^+ exchanger (NHE) exhibits increased activity. Whether this reflects increased numbers of NHE isoform-1 (NHE-1) transporters or increased turnover per molecule has not been established. We have used a specific polyclonal antibody directed toward the C-terminal of NHE-1 to measure NHE-1 content in cultured skin fibroblasts from diabetic patients with (DN) and without (DCON) nephropathy and normal controls (CON). NHE-1 content in fibroblasts from DN subjects was significantly less than that in the other two groups. This suggests that increased NHE activity in diabetic nephropathy is attributed to increased NHE-1 turnover per site rather than increased NHE-1 expression.

THE PATHOGENESIS of nephropathy in type I (insulin-dependent) diabetes depends on a variety of factors including glycemic control,[1] a family history of nephropathy,[2] or a predisposition to essential hypertension, as indicated by increased red blood cell Li^+/Na^+ exchange.[3,4] This latter membrane-transport process may be a surrogate of the physiologic Na^+/H^+ exchanger (NHE),[5] and previous studies have established that leukocytes from patients with diabetic nephropathy (DN) exhibit increased NHE activity similar to that demonstrated in patients with hypertension,[6-8] thus supporting a role for such a predisposition to hypertension in DN. These changes in transport persist despite culture of cells in vitro, and skin fibroblasts from patients with DN[9] are more alkaline than those from normoalbuminuric diabetic controls (DCON) or normal controls (CON), consistent with the increased NHE activity demonstrated at an intracellular pH of 6.5. The persistence of these changes in cells removed from the plasma milieu of patients with DN implies that the dysregulation of NHE in DN is determined by genetic factors. However, the mechanism underlying the increased NHE activity remains obscure and could be due to increased numbers of transporters or increased turnover per site.

Since the description of the sequence for the ubiquitous NHE isoform-1 (NHE-1),[10,11] other members of this family of transporters have been cloned (NHE-2, -3, and -4),[12,13] and it has been suggested that these may be involved in transepithelial transport of Na^+ in gut and kidney epithelia. However, the major NHE isoform in leukocytes or fibroblasts is NHE-1. We have therefore used a specific polyclonal antibody to measure the abundance of NHE-1 in cultured skin fibroblasts from DN, DCON, and CON subjects to determine if the altered NHE activity in DN was due to an increased number of NHE-1 molecules. Our findings suggest that the NHE-1 expression is actually decreased in skin fibroblasts of DN patients, so that increased NHE activity is due to an increased turnover per site.

SUBJECTS AND METHODS

Materials

Nonesterified fatty acid–free bovine serum albumin, glutamine, HEPES, isopropyl β-D-galactopyranoside, and tissue culture medium 199 were obtained from Sigma Chemicals (Poole, Dorset, UK). Dulbecco's modified Eagle's medium (DMEM), minimal essential medium-Eagle, penicillin, and streptomycin were obtained from Gibco, Life Technologies (Uxbridge, Middlesex, UK). Fetal calf serum (FCS) was from Techgen International (London, UK), and the same batch was used throughout the studies. DMEM growth medium contained 10% FCS, 2 mmol · L^{-1} glutamine, 10^5 IU · L^{-1} penicillin, and 100 mg · L^{-1} streptomycin, and was buffered with 24 mmol · L^{-1} $NaHCO_3$ (pH 7.4 in 95% air, 5% CO_2). Protein A Sepharose CL4B, glutathione Sepharose 4B, and the pGEX-2T plasmid were from Pharmacia (Uppsala, Sweden). Hybond C nitrocellulose and enhanced chemiluminescence (ECL) Western blotting reagents were purchased from Amersham International (Amersham, UK). Molecular-weight markers, Lowry protein assay kit, γ-globulin protein standard, *N,N,N′,N′*-tetramethylethylenediamine, and ammonium persulfate were from Bio-Rad (Hertfordshire, UK). Protogel acrylamide solution was obtained from National Diagnostics (Atlanta, GA).

Patients and Skin Fibroblast Culture

The study group consisted of 12 normal healthy nondiabetic control subjects (CON), 12 normoalbuminuric type I diabetic patients (DCON), and 12 diabetic patients with nephropathy (DN). Their clinical characteristics are listed in Table 1. The nephropathy status of patients was determined by the presence of a median albumin excretion rate of three 24-hour sterile urine collections of greater than 300 mg · 24 h^{-1} in a patient with more than a 10-year duration of diabetes and with no heart failure or other renal disease. DCON patients consistently had albumin excretion rates less than 30 mg · 24 h^{-1}, and all subjects were well matched for age, gender, duration of diabetes, and body mass index. Body mass index was calculated from weight and height, and blood pressure readings were obtained after 10 minutes' rest in the supine position. Antihypertensive medication was recorded. A blood sample was analyzed for hemoglobin A_1 ($[HbA_1]$ by high-performance liquid chromatography) and serum creatinine (Jaffé reaction rate method; Hitachi autoanalyzer, BCL, Lewes, East Sussex UK).

Skin fibroblasts were obtained by a punch biopsy from the deltoid region.[9] Initially, the explants were cultured in minimal essential medium-Eagle containing 15% FCS with 2 mmol · L^{-1}

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Table 1. Clinical Characteristics of the Study Groups

	DN	DCON	CON
No. of subjects (male)	12 (7)	12 (6)	12 (7)
Age (yr)	39.1 ± 2.0	38.6 ± 1.8	34.2 ± 2.3
Body mass index (kg · m^{-2})	25.3 ± 1.3	23.8 ± 0.7	23.4 ± 0.7
Duration of diabetes (yr)	26.6 ± 2.0	25.0 ± 2.4	
HbA_1 (%)	9.2 ± 0.2	8.5 ± 0.3	
Albumin excretion rate (mg/24 h)	895 [357-2,547]	9 [2-15]	
Creatinine (μmol/L)	99.4 ± 5.8‡	71.1 ± 2.4	
Blood pressure (mm Hg)			
Systolic	148 ± 4†	129 ± 4	122 ± 3§
Diastolic	90 ± 2*	81 ± 3	78 ± 2§
Antihypertensive therapy (no.)	12	0	0
Retinopathy (no.)			
Minimal	0	4	
Background	2	7	
Proliferative	10	1	

NOTE. Results are the mean ± SEM, except for albumin excretion rates, which are the median [range].

*$P < .01$, †$P < .005$, ‡$P < .001$: *v* DCON.

§$P < .001$ *v* DN.

glutamine and antibiotics. Media were replaced every other day, and cultures were split 1:2 when confluent. After two to three passages, cells were then cultured in DMEM growth medium, which was replaced twice weekly. All cultures were studied between passages five and 10, and care was taken to ensure that all cultures were just confluent at the time of study.

Production of Antisera to Human NHE-1

We obtained a PEX 3 vector (Cambridge Bioscience, Cambridge, UK) bearing nucleotides 2,435 to 2,980 of the C-terminal of human NHE-1 from Professor J. Pouyssegur and Dr C. Sardet of Nice, France.[10,11] The β-galactosidase-NHE-1 C-terminal fusion protein was induced by incubation at 42°C for 2 hours.[10,11] This β-galactosidase fusion protein was electroeluted from gel slices, and a rabbit was immunized by monthly intravenous injections of 100 μg of the protein over 6 months. Immunoglobulin from the serum was partially purified using protein-A Sepharose CL4B beads. The polyclonal antibody used in the present study was derived from one rabbit and denoted as G252.

To ensure specificity of G252, we also constructed another fusion protein (glutathione *S*-transferase NHE-1 C-terminal [GST] fusion protein) using the same sequence described earlier and ligated into the *Eco*RI and *Sma* I sites of a pGEX-2T plasmid.[14] Production of this GST fusion protein was induced with 1 mmol · L^{-1} isopropyl β-D-galactopyranoside with subsequent purification on a glutathione–Sepharose 4B column. The antibody G252 described earlier reacts only with the NHE-1 C-terminal of this GST fusion protein, showing no reactivity toward GST itself. Thus, it was possible to detect any residual nonspecific binding in cell extracts on Western blots when G252 was co-incubated with an excess of GST fusion protein (description to follow).

Western Blotting to Measure NHE-1 Abundance

The procedure for detection of NHE-1 by Western blotting has been previously described.[14] Briefly, fibroblasts from confluent cultures were detached by incubation in HEPES-buffered saline containing EDTA (mmol · L^{-1}: NaCl 140, KCl 5, HEPES 15, glucose 5, and EDTA 1, and bovine serum albumin 1 g/L, pH 7.4 at 37°C). The cells were recovered by centrifugation and washed twice with phosphate-buffered saline. Cells were extracted by adding an equal volume of 125 mmol · L^{-1} Tris, pH 6.8, 5% sodium dodecyl sulfate, 20% glycerol, and 0.004% bromophenol blue solution to cells resuspended in 50 mmol · L^{-1} Tris, pH 7.4, containing NaCl 150 mmol · L^{-1}, EDTA 5 mmol · L^{-1}, phenylmethylsulonyl fluoride 1 mmol · L^{-1}, *o*-phenanthroline 1 mmol · L^{-1} and iodoacetamide 1 mmol · L^{-1}. Extracts were then boiled for 5 minutes. To determine NHE-1 abundance per milligram protein, extracts were analyzed for protein using a modified Lowry assay with γ-globulin as a standard. Aliquots for electrophoresis were reduced by addition of 100 mmol · L^{-1} dithiothreitol, and 10 μg protein was loaded onto each track before resolving the proteins on 7.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis.[15] After electrophoresis, gels were soaked three times (10 minutes each) in chilled transfer buffer (25 mmol · L^{-1} Tris, 192 mmol · L^{-1} glycine, and 20% methanol). Samples were then electroblotted to supported nitrocellulose in transfer buffer containing 0.05% sodium dodecyl sulfate to aid transfer of large molecules[16] for 90 minutes at 100 V using a Bio-Rad transblot cell with plate electrodes and cooling. The membranes were then blocked overnight with 10% low-fat milk powder (Marvel; Premier Beverages, Stafford, UK) in 20 mmol · L^{-1} Tris, pH 7.4, 137 mmol · L^{-1} NaCl, and 0.1% Tween 20 (TBS-Tween). The G252 antibody was then added at 1 μg/mL in TBS-Tween containing 5% Marvel for 2.5 hours. The specificity of serum G252 was validated in experiments by incubating a replicate gel with 1 μg/mL G252 and 2 μg/mL GST fusion protein to neutralize the components of the serum reactive against the C-terminus of NHE-1, as previously described.[14] There was no immunoreactivity in the 103-kd region of these "blocked" blots, where NHE-1 is located. After seven washes with TBS-Tween, the second antibody (1:1,500 dilution of horseradish peroxidase–linked donkey antirabbit Ig) was added for 1 hour. After further washes, nitrocellulose membranes were incubated with ECL developing reagent for 1 minute and then exposed to preflashed x-ray film. The bands of immunoreactivity corresponding to NHE-1 (~ 103 kd) were quantified using a Bio-Rad Imaging densitometer with software for peak identification and area integration (Bio-Rad Laboratories, Hemel Hempstead, Hertfordshire, UK). Molecular-weight markers were visualized using the colloidal gold stain, Aurodye (Amersham International, Amersham, UK). All values for cellular NHE-1 abundance have been expressed relative to a mean value of 1 for the CON group.

Statistics

Data were analyzed using an Oxstat statistics package (Microsoft, Reading, Berks, UK). The mean ± SEM are reported unless otherwise stated, and comparisons were made by ANOVA and Student's *t* test. Spearman's correlation coefficients were also computed, and two-tailed *P* values less than .05 were considered significant.

RESULTS

Clinical characteristics of subjects in the three study groups are listed in Table 1. DN and DCON patients had a similar age, duration of diabetes, and body mass index. DN patients had nonsignificantly higher HbA_1 levels than DCON patients. Systolic and diastolic blood pressures were higher in the DN group (despite antihypertensive therapy) than in DCON or CON subjects (Table 1). All DN patients had evidence of proliferative diabetic retinopathy, as compared with DCON patients, who had mainly minimal or background retinopathy. Serum creatinine levels were higher in the DN group than in the DCON group. Among DCON

patients, all had an albumin excretion rate less than 20 μg/min.

Figure 1 shows a typical Western blot of extracts from fibroblasts for patients in the three groups, DN, DCON, and CON. The NHE-1–specific antibody G252 clearly reacted with a broad protein band of approximately 103 kd. The size and diffuse nature of these protein bands concur with the reported molecular weight of *N*-linked glycosylated NHE-1.[11] Co-incubation of antibody G252 with the GST fusion protein completely abolished immunoreactivity in this molecular-weight range, confirming that the bands detected were NHE-1 (data not shown). Figure 2 shows data derived from a Western blot where different amounts of the GST fusion protein were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and

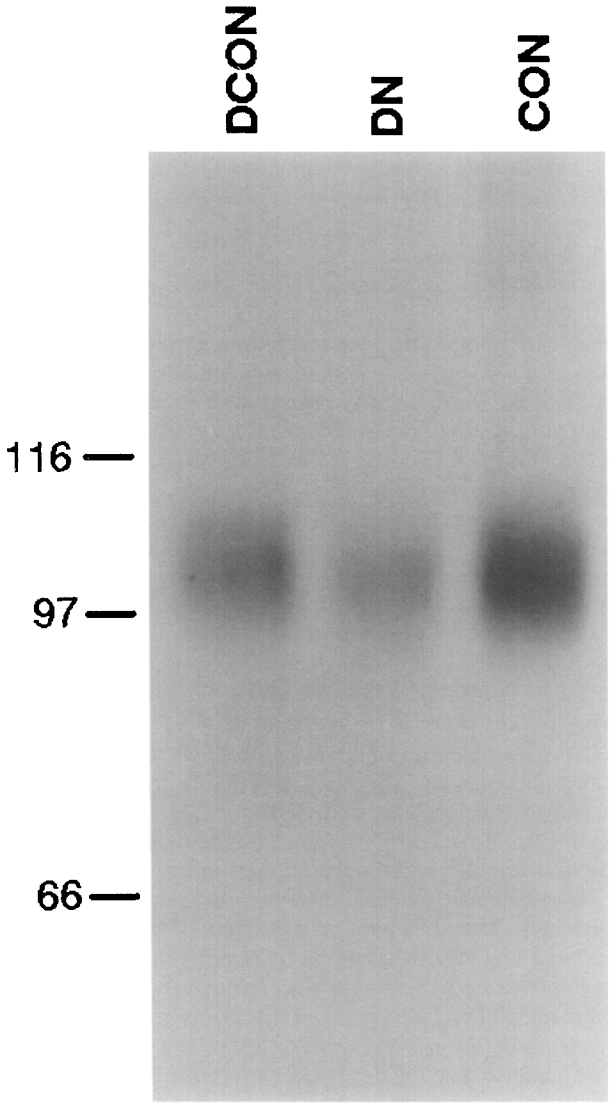

Fig 1. Typical Western blot showing specific immunoreactivity of antibody G252 with a 103-kd protein in cell extracts of fibroblasts from DN, DCON, and CON subjects. Ten micrograms of protein of cell extracts from different patients was loaded in each track. Molecular-weight markers for 66, 97, and 116 kd are shown. NHE-1 abundance in DN cells was less than in cells of the other two groups.

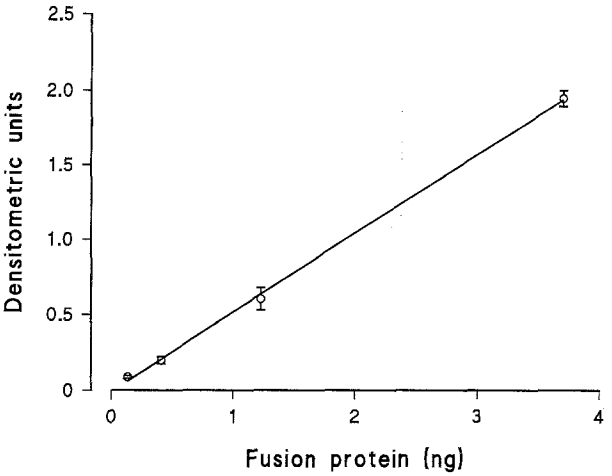

Fig 2. Plot of the amount of GST fusion protein loaded onto a gel against the measured density of the ECL signal on a Western blot, using antibody G252 to detect the C-terminal of NHE-1 present in the GST fusion protein. Means are plotted together with SEM when these exceed the size of the symbol. A linear relationship was demonstrated between amount of fusion protein loaded and density of ECL signal.

detection with antibody G252 was performed with ECL onto preflashed x-ray film. There was a linear relationship between amount of GST fusion protein loaded and density of the signal on the blot. Subsequent blots with fibroblast extracts were performed, and we ensured that the amount of protein loaded produced signals that remained on the linear range of this detection system.

A typical Western blot of extracts from fibroblasts for DN, DCON, and CON subjects is illustrated in Fig 3. Immunoreactivities of extracts for DCON and CON subjects were similar (Fig 3). However, NHE-1 levels were significantly lower in extracts from cells of DN patients (ANOVA F variance ratio = 5.36, $P < .01$) as compared with both DCON and CON subjects (Fig 3: $P < .01$ and $P < .007$, respectively, by Student's t test). Each data point in Fig 3 is the mean of at least two determinations of NHE-1 in separate cultures of cells between passages five and 10.

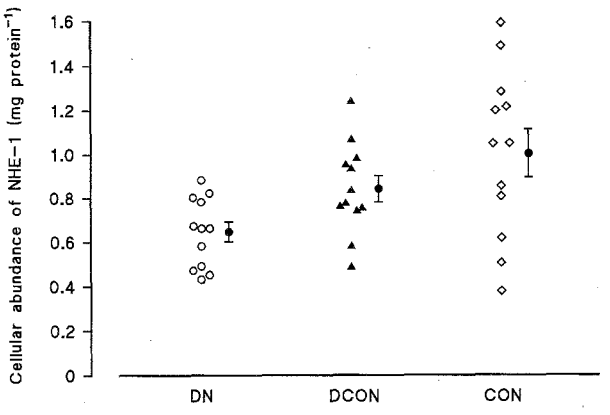

Fig 3. Abundance of NHE-1 expressed per unit of cell-extract protein in the three groups. NHE-1 abundance was significantly less in the DN group as compared with the DCON group ($P < .01$) or CON group ($P < .007$). Data have been normalized to a value of 1 for the CON group.

Although minor molecular-weight heterogeneity between groups was evident with some samples, the means of molecular weights of major bands were not different between the three groups. Thus, it is unlikely that changes in NHE-1 activity were due to gross alterations in N-linked glycosylation of NHE-1.

The decreased NHE-1 levels demonstrated earlier were not due to differences in the passage numbers of cells in the different groups. The passage numbers in DN, DCON, and CON groups averaged 7.2 ± 0.5, 6.9 ± 0.3, and 7.5 ± 0.3, respectively, with no significant differences by ANOVA. Furthermore, NHE-1 abundance in relatively "early"-passage cells (passage number five to seven) was 0.80 ± 0.07, which was not significantly different from that in later-passage cells (passage number eight to 10), where NHE-1 abundance amounted to 0.79 ± 0.06.

In the whole population of subjects, no significant correlations were found between NHE-1 abundance and age ($r_s = -.14$), body mass index ($r_s = -.25$), duration of diabetes ($r_s = -.19$), HbA_1 level ($r_s = -.19$), plasma creatinine level ($r_s = -.22$), or systolic ($r_s = -.27$) and diastolic ($r_s = -.11$) blood pressures. A significant negative correlation was demonstrated between urine albumin excretion and NHE-1 abundance in diabetic subjects ($r_s = -.47, P < .02$).

DISCUSSION

In previous studies, increased activity of NHE has been reported in DN.[6,7,9] In both fibroblasts and leukocytes, NHE-1 is the predominant isoform of NHE.[12,13] Whether the increased activity was due to increased number of NHE-1 molecules or increased turnover per transporter site has never been determined. Knowledge of this would focus research on processes that either control transcription of NHE-1 or alter its activity by posttranslational processes such as phosphorylation.[11]

Measurement of NHE-1 abundance in skin fibroblasts from patients with DN shows that the level of this protein is actually lower than in DCON or CON groups. This suggests that increased transport activity is not due to increased expression of NHE-1 protein, but to an increased turnover number per NHE-1 site. Previous investigation has demonstrated that *N*-linked glycosylation of NHE may affect transporter activity.[17] However, the lack of a consistent difference in molecular weight between the three different groups suggests that a gross change in *N*-linked glycosylation of NHE-1 is unlikely to contribute to the observed differences in transporter activity in nephropathy. In a different population of subjects, we had also determined NHE transport activity and NHE-1 molecular-weight distributions and turnover numbers in Epstein-Barr virus–transformed lymphoblasts,[18] and demonstrated that the increased transport activity in DN cells was due to an increased turnover number of NHE-1, with no changes in NHE-1 numbers per cell. Thus, in both transformed and nontransformed cultured cells from DN patients, increased NHE-1 activity is associated with an increased turnover number of NHE-1 per site. In transformed lymphoblasts from DN patients, this is due to an increased NHE activity with no change in total cellular NHE-1 numbers,[18] whereas in the untransformed adherent fibroblasts, the increased NHE activity is associated with a decreased NHE-1 content. These alterations in NHE-1 content in cells from DN subjects as compared with those from DCON and CON groups may illustrate the differences between the transformed lymphoblast growing in suspension culture and the nontransformed anchorage-dependent fibroblast that exhibits contact inhibition in culture.

These inferences on turnover number are true only if most of NHE-1 is located on the plasma membrane with minimal intracellular compartmentalization. This has been demonstrated in cell-fractionation experiments on both lymphoblasts and fibroblasts.[14,18] The persistence of this phenotype despite culture in vitro suggests that genetic factors play a role in its determination. We cannot exclude the possibility that these differences in NHE-1 protein could have resulted from prior exposure of the skin fibroblasts to a hyperglycemic environment in vivo in DN subjects. However, this may be less likely, since fibroblasts from DCON subjects had NHE-1 protein levels similar to those from CON subjects.

The increased turnover number of NHE-1 with increased NHE activity in DN as compared with DCON subjects may play a role in the pathophysiology of nephropathy and the cardiovascular disease associated with this diabetic complication.[19] Increased cellular NHE activity is associated with stimulation with agents such as growth factors or vascular agonists,[19] so that the increased turnover rate of NHE-1 in DN may be associated with the glomerular mesangial expansion, vascular medial hypertrophy, and left ventricular hypertrophy of these patients. Direct effects of nonenzymatic glycation on the activity of NHE-1 in vivo are currently unknown.

An increased turnover number of NHE-1 has also been inferred from studies on lymphoblasts from hypertensive subjects[20] and on cultured vascular smooth muscle cells of spontaneously hypertensive rats.[21] This supports the hypothesis that patients who develop DN may have a predisposition to hypertension.[3,4] At present, the pathogenesis of the increased turnover number of NHE-1 in both hypertension and DN is uncertain. One possibility is stimulation of NHE-1 by a posttranslational process such as phosphorylation, since this has been demonstrated to increase NHE-1 turnover rate in some cell types.[11,22] This hypothesis remains to be directly tested on immunoprecipitates of NHE-1 in cultured cells from DN patients.

In conclusion, we have demonstrated that cultured skin fibroblasts from DN patients exhibit an increased turnover number for NHE-1, a finding resembling that for lymphoblasts from similar patients. The mechanism underlying this fundamental phenotypic change in DN is not known, but a similar phenotype has been described in cells from hypertensive patients or animals. These cultured cells may serve as models for determining the mechanism leading to this phenotypic change in NHE-1 turnover number in DN.

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