

Abnormal Na^+/H^+ Antiporter Phenotype and Turnover of Immortalized Lymphoblasts from Type 1 Diabetic Patients with Nephropathy

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Abstract

Cellular Na^+/H^+ exchanger (NHE) activity is elevated in type 1 diabetic patients with nephropathy and patients with essential hypertension. The characteristics of this NHE phenotype in hypertension (raised V_{\max} and a lowered Hill coefficient) are preserved in Epstein-Barr virus-transformed lymphoblasts from hypertensive patients. In this study, we have determined NHE kinetics in cultured lymphoblasts from diabetic patients with and without nephropathy, with nondiabetic controls, using fluorometry with the pH indicator 2,7'-bis-(carboxyethyl)-5,6-carboxyfluorescein and estimation of NHE isoform 1 (NHE-1) density with specific polyclonal antibodies. The V_{\max} of NHE was elevated significantly, and the Hill coefficient for internal H^+ binding was lowered in cells from patients with diabetic nephropathy compared with both normal controls and normoalbuminuric diabetic patients. NHE-1 density as measured by Western blotting was similar in all groups. The turnover number of NHE-1 was thus elevated in cells from nephropathy patients. This phenotype in cells from diabetic nephropathy patients resembles that in essential hypertension and suggests that such patients may have a predisposition to hypertension. Moreover, as these changes persist in cultured lymphoblasts in vitro, these cells should provide a cell culture model to further define the basic mechanisms leading to NHE activation in diabetic nephropathy. (*J. Clin. Invest.* 1994. 93:2750-2757.) **Key words:** diabetic nephropathy • pH regulation • Na^+/H^+ antiporter • turnover number • protein blotting

Introduction

Previous work has suggested that several factors may contribute to the pathogenesis of diabetic nephropathy (DN)¹ in type

1 diabetes. These include glycemic control (1), a family history of nephropathy (2), or a predisposition to essential hypertension (HT), as indicated by increased red cell Li^+/Na^+ exchange (3, 4). Another membrane transport abnormality, namely an increased Na^+/H^+ antiport activity, has been reported in a variety of cells from patients with HT (for review see reference 5) and DN (6, 7). In these studies, leukocytes from these two groups of patients exhibited an increased V_{\max} of the Na^+/H^+ antiport. Plasma factors in vivo could have contributed to these changes. Subsequently, we have demonstrated that cultured skin fibroblasts from such patients with DN exhibit an increased Na^+/H^+ antiport activity at a cytosolic pH (pH_i) of 6.5 (8), indicating that transporter characteristics may persist despite in vitro culture. Thus, such ion transport abnormalities may be determined by genetic factors rather than the hormonal milieu present in vivo.

The mechanism of this increased cellular Na^+/H^+ antiport activity in cultured cells from DN has not been defined clearly (8) and may be due to both an increased maximal transport capacity (V_{\max}) and reduced cooperativity (i.e., the Hill coefficient) of the internal H^+ binding site. Moreover, this phenotype has been reported in red cells from both DN (9) and HT (10). Recent work has established that in HT this characteristic phenotype is conserved in cultured Epstein-Barr-immortalized lymphoblasts (11) without any alteration in mRNA transcripts of Na^+/H^+ exchanger isoform 1 (NHE-1). NHE-1 is a member of a growing family of Na^+/H^+ exchangers (12, 13), where NHE-2, -3, and -4 may have a role in transepithelial ion transport. In contrast, NHE-1 is ubiquitous, crucial for control of cell volume and pH_i (12, 13), and may be permissive for cell proliferation (14). Cells of lymphoid origin possess NHE-1 but not the predominantly epithelial isoforms (13, 15, 16), so that changes in Na^+/H^+ exchanger phenotype of these cells may reflect mainly the isoform NHE-1.

In this study, we have therefore used the pH-sensitive fluorophore 2,7'-bis-(carboxyethyl)-5,6-carboxyfluorescein (BCECF) to define the kinetic properties of Na^+/H^+ exchanger in cultured human lymphoblasts from well-matched diabetic subjects with and without nephropathy (DN and DCON, respectively) and nondiabetic controls (CON) to define if the ion transport phenotype established in cells from HT (namely increased V_{\max} and decreased Hill coefficient) is present in DN. This HT phenotype persists in cultured lymphoblasts, implying its dependence on genetic determinants (11). In addition, to define whether the altered V_{\max} was due to a larger number of NHE-1 molecules or to an increased turnover number at each site, we raised specific polyclonal antisera to NHE-1 to estimate its density in these various cell lines. Our results suggest that the Na^+/H^+ exchanger phenotype de-

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1. *Abbreviations used in this paper:* AER, albumin excretion rate; BCECF (-AM), 2,7'-bis-(carboxyethyl)-5,6-carboxyfluorescein (acetoxymethyl ester); CON, nondiabetic controls; DCON, normoalbuminuric diabetics; DN, diabetic nephropathy; GST, glutathione S-transferase; HbA_{1c}, glycosylated hemoglobin; HT, hypertension; NHE-1, Na^+/H^+ exchanger isoform 1; $\text{pH}_{0.5}$, pH_i for half-maximal activation; pH_i , cytosolic pH; r_s , Spearman's correlation coefficient.

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scribed in HT is also present in DN and that the increased V_{\max} was not due to an increased number of NHE-1 molecules, but to an increased turnover at each site.

Methods

Materials. 2,7'-Bis-(carboxyethyl)-5,6-carboxyfluorescein acetoxy-methyl ester (BCECF-AM), nonesterified fatty acid-free BSA, glutamine, nigericin, monensin, adenosine monophosphate, NADPH, NADH, cytochrome *c*, 4-methyl-umbelliferyl- β -D-galactopyranoside, isopropyl β -D-galactopyranoside, and tissue culture medium 199 (TC199) were from Sigma Chemical Company Ltd. (Poole, Dorset, UK). RPMI 1640 culture medium, penicillin, and streptomycin were obtained from GIBCO BRL (Uxbridge, Middlesex, UK). FCS was from Techgen International (London, UK). The TC199 was buffered with 15 mM/liter Hepes to pH 7.4 and contained 1 g/liter BSA. Iscove's modified Dulbecco's medium containing 25 mM Hepes (pH 7.2) was from Gibco Laboratories (Grand Island, NY) and contained 15% FCS (Sigma Immunochemicals, St. Louis, MO), 4 mM L-glutamine, and 10^5 U each of penicillin and streptomycin per liter. Cyclosporin A was from Sandoz Inc. (East Hanover, NJ) and was stored as a stock solution of 20 μ g/ml of medium. Protein A-Sepharose CL4B, glutathione Sepharose 4B, and the pGEX-2T plasmid were from Pharmacia LKB Biotechnology (Uppsala, Sweden). 3 H-3-O-methyl-D-glucose, Hybond C nitrocellulose, and enhanced chemiluminescence Western blotting reagents were purchased from Amersham International (Amersham, UK).

Patients and lymphoblast culture. The study group consisted of 23 normal healthy CON subjects, 17 DCON patients, and 17 DN patients. The diabetic patients were selected from among those previously described in reports of a case-control study of determinants of the complications of type 1 diabetes (17). That study included 162 patients who had developed type 1 diabetes before the age of 21, were Caucoid, and attended the Joslin Clinic between 1967 and 1972, within 2 yr after the diagnosis of diabetes. They were examined in 1986 to 1988 regardless of whether they were under the care of the Joslin Clinic. The nephropathy status of patients was determined by a timed collection of urine (3 h) as described previously (17) and urinary albumin measured by radioimmunoassay (Diagnostic Products Corp., Los Angeles, CA). In the group of 162 patients, there were 43 with overt proteinuria (albumin excretion rate [AER] above 300 μ g/min, including 16 with renal failure) and 61 without nephropathy (AER < 20 μ g/min) despite 15–21 yr of diabetes. In 1991, immortalized lymphoblast cell lines were established for two randomly selected groups of these patients, 17 of the 43 with overt proteinuria and 17 of the 61 without DN. At the same time, immortalized lymphoblast cell lines were established from 23 healthy nondiabetic individuals.

The clinical characteristics of the study groups are shown in Table I. Clinical and biochemical data obtained during the 1986–1988 assessment and biochemical data from 1991 to 1992 are used in this report. Glycosylated hemoglobin (HbA_{1c}) and serum creatinine were determined by methods described previously (17). All subjects had measurements of blood pressure after 10 min of rest, and height and weight were recorded to determine the body mass index (kilograms per square meter). Any antihypertensive medication that patients were receiving was recorded. The presence of retinopathy was determined by fundus photography of seven standard fields of the retina and graded as minimal, background, or proliferative retinopathy.

Peripheral blood lymphocytes were isolated from subjects using density gradient centrifugation using LeucoPREP tubes (Becton Dickinson, Lincoln Park, NJ). Separated B lymphocytes were suspended in 2 ml of Iscove's modified Dulbecco's growth medium and infected with 0.5 ml of EBV. Into each tissue culture flask, 0.5 ml of cyclosporin A was also added, as this increases the efficiency by which permanent lymphoblastoid cell lines are established (18), since T cells have been implicated in the spontaneous regression of EBV-transformed cultures

Table I. Clinical Characteristics of the Study Groups

	DN	DCON	CON
Number (male)	17 (9)	17 (6)	23 (10)
Age (yr)	33.2±1.1	35.2±1.1	34.3±2.2
Body mass index (kg/m ²)	25.2±1.7	22.9±0.9	24.9±1.0
Duration of diabetes (yr)	18.3±0.7	18.1±0.4	
Insulin dose (IU/d)	48±4	51±4	
HbA _{1c}	12.5±0.6*	10.5±0.4	
Albumin excretion rate (μ g/min)	2612±689 [‡]	7.5±0.8	
Creatinine (μ mol/liter)	165±40 [§]	71±4	
Systolic BP (mmHg)	147±5	112±3	113±3
Diastolic BP (mmHg)	93±4	73±2	73±2
Antihypertensive therapy	9	0	0
Retinopathy:			
Minimal	0	6	
Background	3	9	
Proliferative	14	2	

Results are means ± SEM. [§] $P < 0.05$, * $P < 0.01$, [‡] $P < 0.001$, all compared with DCON. ^{||} $P < 0.001$ compared with CON.

(19). Cells were incubated at 37°C for 7 d and were refed with 2 ml of the complete growth medium. When large clumps appeared (usually 2–3 wk after starting the cultures), the cells were fed by doubling the volume of the complete growth medium every 3 to 4 d until they reached a volume of 50 ml with an approximate density of 10^6 cells/ml. The immortalized cells were harvested by centrifugation (800 rpm, 7 min), suspended again in complete growth medium containing 10% DMSO, and stored in vials in liquid nitrogen. The time from initiation of a culture to freezing was variable; on average it was about 3–4 wk.

The immortalized cell lines used for this study were recovered from liquid nitrogen storage and cultured in RPMI 1640 growth medium containing 15% FCS, 11.1 mM glucose, 1 mM glutamine, 10^5 IU/liter penicillin, and 100 mg/liter streptomycin. The same batch of serum was used throughout the study. The RPMI 1640 medium was buffered with 24 mM NaHCO₃ (pH 7.4, in 95% air, 5% CO₂). Cell density was determined daily on a Coulter counter (model ZM; Coulter Electronics, Luton, UK) and was maintained between 0.25 and 0.75×10^6 /ml. The rate of lymphoblast proliferation was determined by measuring cell number every day and fitting the data with the following equation: $N = N_0 e^{kt}$, where N is the cell density at an elapsed time t , N_0 the initial cell density, and k the time constant for cell proliferation.

Measurement of pHi and Na⁺/H⁺ antiport activity. Lymphoblasts (15×10^6 cells) were incubated with BCECF-AM (10 μ M in TC199) at 37°C for 30 min. The cells were then washed extensively three times with TC199 and left in this medium for 30 min at room temperature before any measurements were made, to ensure complete deesterification of the BCECF-AM. Experiments were performed without a prior period of serum withdrawal.

Measurements of pHi have been described in detail previously (6–8). Briefly, resting pHi was determined in both Hepes-buffered saline ([mM] NaCl 140, KCl 5, CaCl₂ 1.8, MgSO₄ 0.8, glucose 5, Hepes 15, BSA 1 g/liter, pH 7.4, at 37°C) and also in NaHCO₃-buffered saline ([mM] NaCl 126, NaHCO₃ 24, KCl 5, CaCl₂ 1.8, MgSO₄ 0.8, glucose 5, BSA 1 g/liter, pH 7.4, at 37°C when equilibrated with 95% air, 5% CO₂). The two excitation wavelengths were set at 500 and 439 nm (slit widths 5 nm) with emission at 530 nm (slit width 5 nm) in a single photon-counting dual-excitation fluorometer (Photon Technology International Deltascan, South Brunswick, NJ). Autofluorescence was subtracted from fluorescence readings before calculation of 500/439 ratios. The 500/439 ratios were converted subsequently to pH values by constructing a calibration curve with pHi clamped to 6.0, 6.2, 6.4,

6.6, 6.8, 7.2, and 7.8 in isotonic KCl buffers ([mM] KCl 140, CaCl₂ 1.8, MgSO₄ 0.8, glucose 5, Hepes 15, with 5 μ M nigericin and 5 μ M monensin) as described previously (6–8).

All measurements of buffering and H⁺ efflux were performed at 37°C. Lymphoblasts were clamped to pH_i ranging from 6.0 to 6.8 by the monensin and nigericin pH_i clamping procedure described above. The ionophores were then scavenged using BSA (1 g/liter). The intracellular buffering capacity was then determined in duplicate at that pH_i using NH₄Cl (10 mM at pH_i 6.4, 6.6, and 6.8, 30 mM at pH_i 6.2, and 50 mM at pH_i 6.0) in the KCl buffer containing BSA. Isotonicity was maintained by substituting the NH₄⁺ for K⁺ ions in these buffers. These concentrations of NH₄⁺ ensured that the pH_i changes resulting from the NH₄Cl pulse exceed 0.4, since changes in pH_i less than this at pH_i 6.0 could lead to an overestimation of buffering capacity and hence H⁺ efflux. The efflux of H⁺ purely from the internal to external pH gradient (pH_i 6.0–6.8, external pH 7.4) was then measured by addition of buffer containing 140 mM *N*-methyl-D-glucamine chloride. H⁺ efflux in this Na⁺-free medium was identical to that when cells were inhibited with 10 μ M ethyl-isopropyl amiloride. The sensitivity of H⁺ efflux to ethyl-isopropyl amiloride (12, 13, 15, 16) indicated that the major NHE isoform in these cells is likely to be NHE-1. Total H⁺ efflux was then measured in Hepes-buffered saline. The rate of change of pH_i due to the Na⁺/H⁺ antiport is the difference between the slope of the efflux measured in Hepes-buffered saline and that measured in buffered *N*-methyl-D-glucamine chloride. All measurements of flux were done in triplicate, using slopes of 100 pH_i readings against time from the initial 20 s of records, and the Pearson correlation coefficients for slope determinations routinely exceeded 0.99. Flux rates of the Na⁺/H⁺ antiport (millimolar per minute) were obtained as the product between these rates of change of pH_i and the intrinsic buffering capacity. Measurements of pH_i and Na⁺/H⁺ antiport activity in the lymphoblasts were performed blind so that the origin of the cells was not known until the completion of the study.

Na⁺/H⁺ antiport fluxes were fitted to the logistic (Hill) equation to obtain values for V_{\max} , the pH_i for half-maximal activation (pH_{0.5}), and the apparent Hill coefficient for the internal H⁺ binding site of the antiport. A Hill coefficient > 1 indicates positive cooperativity for H⁺ binding. The computer program used to derive these parameters was a recursive nonlinear least squares algorithm (P-fit; Biosoft Corporation, Cambridge, UK). These parameters were also checked using another nonlinear optimization algorithm that we described previously (20) that uses a pattern search method to minimize the errors between calculated and observed flux values. Both algorithms produced very similar results for the different parameters V_{\max} , pH_{0.5}, and the Hill coefficient.

Production of antisera to human Na⁺/H⁺ antiporter. A PEX 3 vector (EMBL, Genofit) bearing nucleotides 2435–2980 of the human Na⁺/H⁺ antiporter NHE-1 was obtained from Prof. J. Pouyssegur and Dr. C. Sardet (University of Nice, France) (21). Induction of the β -galactosidase–NHE-1 carboxyl-terminal fusion protein was performed by incubation at 42°C for 2 h as described previously (21). The β -galactosidase fusion protein was electroeluted from gel slices, and two rabbits were immunized by monthly intravenous injections of 100 μ g of the β -galactosidase fusion protein over a period of 6 mo. The immunoglobulins were partially purified using protein A–Sepharose CL4B beads. The polyclonal antibody used in this study is from one rabbit and denoted as G252.

A glutathione S-transferase (GST) NHE-1 carboxyl-terminal fusion protein was also constructed, ligating the same nucleotides to the SmaI site of a pGEX-2T plasmid. Production of this GST fusion protein was induced with 1 mM isopropyl β -D-galactopyranoside with subsequent purification on a glutathione–Sepharose 4B column. The antibodies described above react only with the NHE-1 carboxyl terminus of this GST fusion protein, showing no reactivity toward GST itself.

Protein blotting to measure Na⁺/H⁺ antiport density. To determine NHE-1 density per cell, extracts from known numbers of cells (determined on a Coulter counter) were resolved on 7.5% SDS-PAGE gels. Cells were resuspended in 50 mM Tris, pH 7.4, containing 150

mM NaCl, 5 mM EDTA, 1 mM phenyl methyl sulphonyl fluoride, 1 mM *o*-phenanthroline, and 1 mM iodoacetamide. An equal volume of buffer (composed of 0.125 mM Tris, pH 6.8, 5% SDS, 20% glycerol, 0.004% bromophenol blue) was added, and the extract was boiled for 10 min. After electrophoresis, gels were soaked three times (10 min each) in prechilled transfer buffer (25 mM Tris, 192 mM glycine, and 20% methanol) followed by electroblotting to supported nitrocellulose. The membrane was blocked overnight with 10% low fat milk powder (Marvel) in 20 mM Tris, pH 7.4, 137 mM NaCl, and 0.1% Tween 20 (TBS-Tween) and incubated with 1 μ g/ml of G252 antibody in 5% Marvel in TBS-Tween for 2.5 h. The specificity of serum G252 was validated in every experiment by incubating a replicate gel with 1 μ g/ml G252 plus 2 μ g/ml GST fusion protein to neutralize the components of the serum reactive against the carboxyl terminus of NHE-1. These “blocked” blots show no immunoreactivity in the 92–114-kD region, where NHE-1 is located. After extensive washes with TBS-Tween, the second antibody (1:1,500 dilution of horseradish peroxidase-linked donkey anti-rabbit Ig) was added for 1 h. After further washes, the nitrocellulose membrane was incubated with enhanced chemiluminescence developing reagent for 1 min and then exposed to preflashed x-ray film. The bands corresponding to NHE-1 (~ 92–114 kD) were quantitated using a laser densitometer and a software package (LKB Ultrascan XL and Gelscan XL, respectively; Pharmacia LKB Biotechnology). Molecular weight markers were visualized using the colloidal gold stain, Aurodye. To determine absolute numbers of NHE-1 per cell, a dilution series of GST NHE-1 carboxyl-terminal fusion protein was loaded alongside cell samples on 9% acrylamide gels. These were electroblotted and developed as above. We have assumed that the affinities of the G252 antibody for the NHE-1 and for the GST fusion protein are the same. Construction of a standard curve of density and amount of fusion protein loaded enabled the number of NHE-1 molecules per cell to be estimated, using a value of 6.022×10^{23} as Avogadro’s constant.

Since the V_{\max} of the Na⁺/H⁺ exchanger was expressed in millimoles per liter of cell water per minute, and NHE-1 density was expressed as sites per cell, determination of turnover numbers required the measurement of the intracellular water space. This was determined using ³H-3-*O*-methyl-D-glucose (22), which is a nonmetabolizable hexose that equilibrates into cellular water. Subsequent efflux was inhibited by 0.25 mM phloretin when the cells were washed free of extracellular label. Cell number was determined on a Coulter counter, as described earlier.

Subcellular fractionation of lymphoblast membranes. Lymphoblasts (50–60 $\times 10^6$ cells) were washed twice in Hepes-buffered saline and then once in sucrose buffer ([mM] sucrose 250, EDTA 1, Tris 1, pH 7.2), recovering the cells into 2 ml of homogenization buffer composed of (mM) sucrose 250, EDTA 1, Tris 1, phenylmethyl sulphonyl fluoride 1, and leupeptin 5 μ M, pH 7.2. Then, the cells were disrupted using nitrogen cavitation as described previously (23, 24). Briefly, the 2 ml of cell suspension was pressurized in the nitrogen cavitation apparatus (Kontes, Vineland, NJ) at a pressure of 10 bar for 15 min at 4°C. The suspension was then slowly depressurized through the outlet, resulting in rupture of over 99% of the cells. The homogenate was centrifuged at 600 g for 10 min at 4°C to obtain a postnuclear fraction. Then, this was layered onto a self-generated gradient of 30% Percoll in homogenization buffer at 27,000 g for 60 min at 4°C (25). By centrifugation of an identical tube containing colored density marker beads, the following fractions were collected: the cytosolic fraction, above the interface with the Percoll; fraction i at a density of 1.042 g/ml; fraction ii at 1.05 g/ml; fraction iii at 1.056 g/ml; fraction iv between 1.069 and 1.083 g/ml; and fraction v between 1.102 and 1.128 g/ml. Proteins in all fractions were determined using a fluorometric method using fluorescamine as described previously (24), and 25 μ g of protein from each fraction was loaded onto 7.5% SDS gels and analyzed by Western blotting as described above. The marker enzymes used to identify the membranes in these fractions included lactate dehydrogenase for the cytosol (24), 5'-nucleotidase for plasma membranes (26), rotenone-insensitive NADPH-cytochrome *c* reductase for endoplasmic reticu-

lum (27), and β -galactosidase for lysosomes with 4-methyl-umbelliferyl- β -D-galactopyranoside as substrate (24).

Statistics. Data were analyzed using an Oxstat statistics package (Microsoft Corporation, Reading, Berks, UK). Means and SEM are reported, and comparisons were by ANOVA and Student's *t* test. Two-tailed *P* values < 0.05 were taken as significant.

Results

Clinical characteristics of the study groups are presented in Table I. Type 1 diabetic patients with and without nephropathy had similar ages, duration of diabetes, and body mass index and required similar daily doses of insulin. However, those with DN had poorer glycemic control when compared with DCON, as evidenced by higher mean HbA_{1c}.

Patients classified as having DN had advanced renal complications. This included two patients on hemodialysis, two with renal transplants, and the remaining with overt proteinuria. Among the DCON patients, all had an AER < 20 μ g/min. Nine of the group with DN had a parental history of hypertension compared with four of the DCON group. The two diabetic groups also differed with regard to systolic and diastolic blood pressure (Table I). All diabetic patients had evidence of diabetic retinopathy, although a higher proportion of those with DN had severe proliferative retinopathy. Blood pressures in the DCON group were similar to that in the CON group. Serum creatinine levels were higher in the DN group than in the DCON group.

The rate of lymphoblast proliferation was very similar among all three groups of subjects (mean \pm SEM $k \times 10^{-3} \text{ h}^{-1}$ in subjects with DN was 15.53 ± 0.71 , in DCON 13.95 ± 0.57 , and in CON $14.15 \pm 0.82 \text{ h}^{-1}$). The pH_i measured in Hepes-buffered saline was also very similar in all three groups of subjects (Table II). In NaHCO₃-buffered saline, there were no significant differences between the three groups of subjects (Table II), although pH_i values were higher in NaHCO₃-buffered saline in cells from patients with DN ($P < 0.003$) and CON ($P < 0.001$). Intrinsic buffering capacities in the groups of subjects were similar (Table II). The buffering in NaHCO₃-containing saline was significantly higher than in Hepes-buffered saline ($P < 0.001$) with no differences between groups (Table

Table II. Cytosolic pH, Buffering Measurements, and NHE Kinetic Data of Lymphoblasts from CON, DCON, and DN

	DN	DCON	CON
Cytosolic pH			
pH _i in Hepes buffer	7.13 \pm 0.03	7.17 \pm 0.03	7.13 \pm 0.02
pH _i in NaHCO ₃ buffer	7.19 \pm 0.02	7.20 \pm 0.03	7.19 \pm 0.02
Buffering capacity (mmol/liter per pH unit)			
Buffering capacity at resting			
pH _i in Hepes buffer	16.58 \pm 0.91	14.66 \pm 0.94	18.72 \pm 1.11
Buffering capacity at resting			
pH _i in NaHCO ₃ buffer	27.07 \pm 1.19	25.84 \pm 2.41	30.56 \pm 1.44
NHE kinetic data			
V_{max} (mM/min)	22.07 \pm 1.33*	16.28 \pm 0.78	15.31 \pm 0.88
Hill coefficient	1.94 \pm 0.06*	2.59 \pm 0.13	2.32 \pm 0.08
pH _{0.5}	6.37 \pm 0.03	6.36 \pm 0.03	6.29 \pm 0.03

Results are means \pm SEM. No significant differences were found among the three groups of subjects in cytosolic pH or buffering measurements, but the V_{max} was significantly higher and the Hill coefficient lower in DN compared with CON and DCON. * $P < 0.001$ compared with DCON and CON.

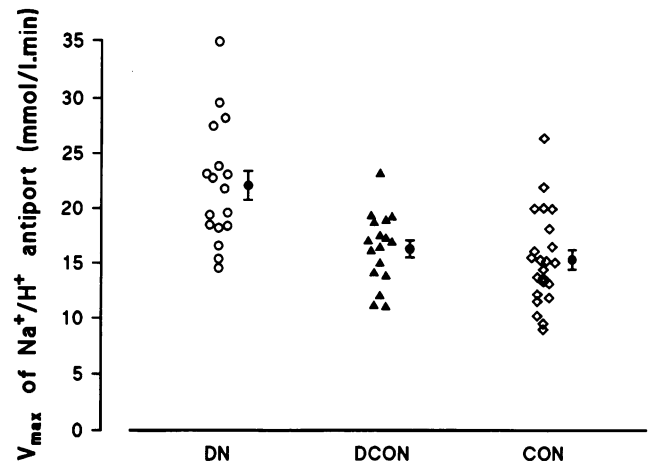


Figure 1. V_{max} of the Na⁺/H⁺ antiport in lymphoblasts from normal controls (CON) and diabetic patients with DN and without nephropathy (DCON). Means and SEM are plotted. The V_{max} of lymphoblasts from DN ($22.07 \pm 1.33 \text{ mM/min}$) was elevated significantly compared with both CON ($15.31 \pm 0.88 \text{ mM/min}$, $P < 0.001$) and DCON ($16.28 \pm 0.78 \text{ mM/min}$, $P < 0.001$).

II). Intrinsic buffering capacities measured at the different pH_i studied (range 6.0–6.8) were similar in all three groups (data not shown).

H⁺ efflux rates due to the Na⁺/H⁺ antiport, at pH_i 6.0, between groups differed when compared by ANOVA ($P < 0.001$). These H⁺ efflux rates were significantly higher in lymphoblasts from patients with DN ($17.14 \pm 0.86 \text{ mM/min}$) compared with CON ($13.69 \pm 0.82 \text{ mM/min}$, $P < 0.001$) or DCON ($14.79 \pm 0.73 \text{ mM/min}$, $P < 0.05$). The kinetics of the lymphoblast Na⁺/H⁺ antiporter was examined in greater detail. The pH_{0.5} was very similar between all three groups (Table II). The V_{max} of the Na⁺/H⁺ antiport was elevated very significantly in the cells from patients with DN ($F = 12.78$, $P < 0.001$ by ANOVA) compared with both CON and DCON subjects (Fig. 1 and Table II, $P < 0.001$ for both comparisons by Student's *t* test). In this subgroup of DN patients, the Hill coefficient was also significantly lower than the other two groups ($F = 10.84$, $P < 0.001$ by ANOVA; Fig. 2 and Table II, $P < 0.001$ for both comparisons), suggesting a reduced positive cooperativity for H⁺ binding in the DN group. These changes in V_{max} or the Hill coefficient in DN cannot be attributed to the renal impairment or immunosuppressive therapy of these patients since these abnormalities were still present and significant ($P < 0.001$) when the four patients on dialysis or who had undergone transplantation were excluded from the analysis.

In the whole population of subjects, the resting pH_i was correlated to the pH_{0.5} of the Na⁺/H⁺ antiport (Spearman's correlation coefficient [r_s] = -0.33 , $P < 0.01$). The Hill coefficient was negatively correlated with the V_{max} ($r_s = -0.45$, $P < 0.001$). There were no significant correlations between age, body mass index, duration of diabetes, insulin dose, HbA_{1c} levels, and any of the pH_i or Na⁺/H⁺ antiport activity measurements. However, systolic and diastolic blood pressure correlated positively with the V_{max} of the Na⁺/H⁺ antiport (systolic BP vs V_{max} , $r_s = 0.54$, $P < 0.001$; diastolic BP vs V_{max} , $r_s = 0.40$, $P < 0.002$) and negatively with the Hill coefficient (systolic BP vs Hill coefficient, $r_s = -0.41$, $P < 0.002$; diastolic BP vs Hill coefficient, $r_s = -0.34$, $P < 0.01$).

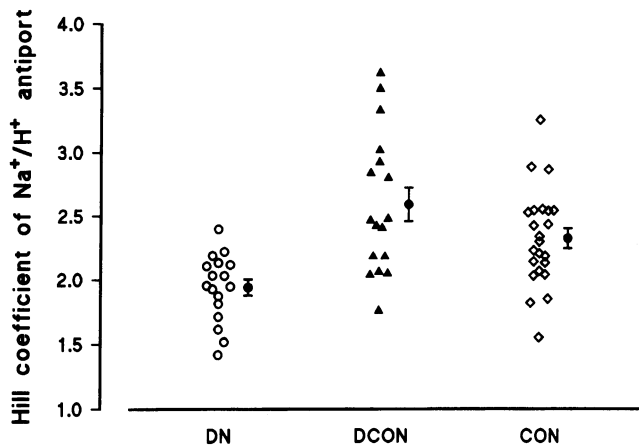


Figure 2. Hill coefficient of the internal H^+ binding site of the Na^+/H^+ antiporter in lymphoblasts from CON, DN, and DCON. Means and SEM are plotted. Hill coefficients of lymphoblasts from DN (1.94 ± 0.06) were significantly lower than those of CON (2.32 ± 0.08 , $P < 0.001$) and DCON (2.59 ± 0.13 , $P < 0.001$).

A typical Western blot of lymphoblast cell extracts obtained from equal numbers of cells from the different cell lines is shown in Fig. 3 A. The NHE-1-specific antibody G252 clearly reacted with protein bands ranging in molecular masses from 92 to 114 kD, the major bands being ~ 96 and 102 kD. The size of these protein bands concur with the reported molecular mass of N-linked glycosylated NHE-1 (21). Incubation of G252 antibody with the GST NHE-1 carboxyl-terminal fusion protein abolished this specific immunoreactivity (Fig. 3 B). By co-loading known amounts of GST fusion protein onto gels and exposing the nitrocellulose membranes to preflashed film, a linear relation was obtained between fusion protein band density and amount of protein. From these calibration curves, we could determine the exact total amount of NHE-1 protein present in the bands (92–114 kD) of the lymphoblast extracts. Using Avogadro's number, the NHE-1 immunoreactive sites were very similar between all three groups of subjects (Table III). Thus, the documented elevated V_{max} in the DN group could not be due to elevated numbers of NHE-1 molecules.

Homogenization of lymphoblasts using nitrogen cavitation with subsequent isopycnic centrifugation through Percoll enabled fractionation of lymphoblast membranes. The enzyme markers were enriched (when compared with the postnuclear supernatant) in the various fractions as follows: the cytoplasmic fraction was enriched 1.6-fold with the cytosol marker lactate dehydrogenase; fraction i was enriched 3.3-fold in 5'-nucle-

Table III. Estimates of NHE-1 Density and Turnover Number from Western Blots Performed on the Lymphoblast Extracts of CON, DCON, and DN

	DN	DCON	CON
NHE-1 density (sites/cell)	22482 \pm 759	21489 \pm 720	22927 \pm 557
NHE-1 turnover rate (cycles s^{-1})	9845 \pm 596	7631 \pm 433*	6695 \pm 428 [†]

* $P < 0.005$ and [†] $P < 0.001$, compared with DN group.

otidase; fraction ii 2.7-fold in NADPH-cytochrome *c* reductase; and fraction v 3.6-fold in β -galactosidase. Fig. 4 shows that most (98%) of the NHE-1 immunoreactivity was recovered in fraction i which was enriched with the plasma membrane marker 5'-nucleotidase. The specific reactivity of NHE-1 in fraction i relative to the postnuclear supernatant ranged from 4.4 to 6.4 in three experiments. The various bands present in the postnuclear supernatant were all present in this fraction, with no evidence of compartmentalization of particular bands within the cell. Fraction ii contained $\sim 4\%$ of total 5'-nucleotidase activity and 2% of NHE-1 immunoreactivity. The small amount of NHE-1 in this fraction may reflect minor contamination of endoplasmic reticulum with plasma membranes.

The predominant plasma membrane localization of NHE-1 enabled an estimate of its turnover number in the different cell lines, since V_{max} (in millimoles per liter of cell water per minute) and NHE-1 density per cell were known. Moreover, NHE-1 is the predominant isoform present in lymphoid cells (13, 15, 16). This is supported further by the sensitivity of lymphoblast Na^+/H^+ exchange to ethyl-isopropyl amiloride. Cellular volumes were determined using the 3H -3-O-methyl-D-glucose technique (22). Turnover numbers were calculated to be significantly higher in DN compared with DCON or CON (Table III) with no difference between DCON and CON.

Discussion

Several studies have reported alterations in Na^+/H^+ exchanger kinetics in a variety of cells from HT patients (5, 10, 11, 28, 29). This phenotype of a raised V_{max} and lowered Hill coefficient is conserved in immortalized lymphoblasts from HT (11), indicating the importance of genetic factors in determining these changes. No differences were demonstrated in the NHE-1 mRNA transcripts of HT cell lines (11), although no direct measurements of NHE-1 protein were performed.



Figure 3. (A) A typical western blot showing specific immunoreactivity of antibody G252 with a 92–114-kD protein in cell extracts from lymphoblasts from different subjects (DN, DCON, and CON). Extracts from 10^6 cells from different patients were loaded in each track. The molecular mass marker for 97 kD is shown. There were no significant differences in NHE-1 density between DN, DCON, and CON. (B) The specific immunoreactivity shown in A was abolished completely by addition of GST-NHE-1 fusion protein during the G252 incubation, leaving non-specific bands.

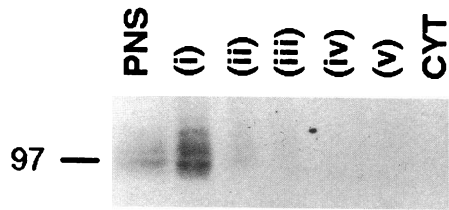


Figure 4. Lymphoblasts were homogenized by nitrogen cavitation, and fractions were isolated after centrifugation through Percoll. From the postnuclear supernatant and each fraction, 25 μg protein was loaded onto each track of 7.5% SDS gels and blotted as described. The molecular mass marker for 97 kD is shown. Fraction i was enriched with NHE-1 and 5'-nucleotidase when compared with postnuclear supernatant (PNS). Fraction ii was enriched in NADPH-cytochrome *c* reductase and v in lysosomal β -galactosidase. No NHE-1 was present in the cytosolic fraction (CYT).

Patients with DN have a predisposition to HT, as evidenced by another membrane transport marker, elevation of the red cell Li^+/Na^+ exchange (3, 4, 28, 29). In previous studies, cultured skin fibroblasts from DN patients showed a higher resting pH_i and a raised Na^+/H^+ antiport activity when pH_i was clamped at 6.5 but not at 6.2 (8). However, we had not performed a full kinetic analysis of Na^+/H^+ exchange in the fibroblasts, so that the reason for this increased activity, at pH_i 6.5, was uncertain. Moreover, a true V_{max} may not have been achieved in the DN fibroblasts at pH_i 6.2. It is possible that these findings in fibroblasts may have resulted from an increased V_{max} and reduced Hill coefficient for internal H^+ binding in DN patients, as similar changes in these parameters in phorbol ester-treated lymphoblasts from hypertensives (11) led to a difference in Na^+/H^+ antiport activity, at pH_i 6.8–7.0, with a less obvious difference at pH_i 6.5 before the activation curves diverge towards their respective V_{max} values (the phorbol ester treatment may have shifted the activation curves towards more alkaline values than described in our study).

In the present study on lymphoblasts, the resting pH_i values were similar in all three groups of subjects. The pH_i values reported for these lymphoblasts are more alkaline than those found in skin fibroblasts (8), and it is likely that in the quiescent state fibroblasts may still exhibit some Na^+/H^+ exchange since their pH_i values were below 7.0. H^+ binding to the internal modifier site of the Na^+/H^+ antiport exhibits positive cooperativity as evidenced by the Hill coefficient values > 1 in this study, although the cooperativity may not be as great in cells from patients with DN. Thus, above a pH_i of 7.0, the Na^+/H^+ antiport would be effectively switched off, so that no differences in pH_i would be evident. Furthermore, pH_i is dependent on metabolic acid production and not just Na^+/H^+ exchanger activity. In the presence of 24 mM NaHCO_3 (pH 7.4, with 5% CO_2), there was no significant difference between the resting pH_i of lymphoblasts from the DN and DCON patients and CON subjects. This could have been because of enhanced buffering capacity in the presence of HCO_3^- and/or enhanced activity of anion exchangers as described recently by Alonso et al. (30) in hypertensive patients.

To study the kinetics of the Na^+/H^+ antiport in isolation, the effects of $\text{Cl}^-/\text{HCO}_3^-$ exchange were excluded by performing H^+ efflux studies in the nominal absence of HCO_3^- (Hepes-buffered media). The cells were not subjected to a period of

serum withdrawal, since they tended to deteriorate and variable Na^+/H^+ antiport fluxes were produced. Since the growth rates of the cells from different groups were so similar, it is likely that the proportion of cells undergoing mitosis is also similar between groups. The $\text{pH}_{0.5}$ for NHE activation was similar among the different groups, although this value in lymphoblasts was lower than that reported previously in lymphocytes (31) and may reflect the proliferation of these transformed cells. The lymphoblasts from patients with DN showed a very significant increase in the V_{max} and a lowered Hill coefficient for internal H^+ binding compared with both CON and DCON patients. In a previous report (6), leukocytes freshly isolated from patients with DN similarly had an increased V_{max} compared with the other two groups, although we did not observe a lowered Hill coefficient in freshly isolated leukocytes from patients with DN. Thus, it is uncertain if the differences in Hill coefficient in the present study arose because lymphoblasts rather than neutrophils were studied or whether plasma factors *in vivo* could alter this particular parameter of the transporter. Roskopf et al. (11) have described a lowered Hill coefficient in phorbol ester-treated lymphoblasts from hypertensives, suggesting a role for phosphorylation in altering this parameter.

However, the finding of an increased V_{max} in lymphoblasts and neutrophils (6) is consistent and suggests that the differences in this kinetic property of the Na^+/H^+ antiporter may have persisted despite culturing of the transformed lymphoblasts *in vitro* in defined RPMI growth medium with 11.1 mmol/liter glucose. We cannot exclude the possibility that these differences could have resulted from prior exposure of the lymphocytes to a hyperglycemic environment in the DN subjects, but since the lymphoblasts from normoalbuminuric DCON subjects had kinetic parameters similar to those from nondiabetic controls, this explanation may seem less likely. These changes in the kinetic properties of the Na^+/H^+ antiport were not due to changes in cytosolic buffering, as intrinsic and HCO_3^- buffering were similar in all three groups of subjects. Moreover, these abnormalities cannot be attributed to the renal impairment of DN patients, as they remained even after the uremic subjects were excluded. Furthermore, we had demonstrated previously that the Na^+/H^+ antiport activity of leukocytes from patients with chronic renal failure was similar to that in normal nondiabetic controls (32). The changes in ion transport in DN were not associated with altered proliferation rates of the lymphoblasts, in contrast to another study in lymphoblasts from HT subjects (11). In that study, the cells were selected from HT patients who possessed increased Na^+/H^+ antiport activity (11) and were compared with normotensive controls with low Na^+/H^+ antiport activity, whereas our study patients were not subjected to any selection bias. The fluxes reported in that study (11) were higher than those in our study, and these differences may have resulted from different culture conditions (e.g., serum) or the use of phorbol esters (11).

These kinetic abnormalities of the lymphoblast Na^+/H^+ antiport in DN, namely a lowered Hill coefficient and a higher V_{max} , have been reported previously in red cells from patients with DN (9) and HT (10, 28). We and others have also reported previously a significantly higher V_{max} for the Na^+/H^+ antiport in a variety of cells from patients with HT with a lower Hill coefficient (for reviews see references 5, 11, 28, 29). It is interesting that the V_{max} and Hill coefficients of Na^+/H^+ exchange in the present study correlated positively and negatively, respectively, with blood pressures and not with other

indices of glycemic control. Taken in conjunction with other studies where markers for a predisposition to hypertension such as elevated red cell Li^+/Na^+ exchange have been described in DN (3, 4, 28, 29), the present study further supports the hypothesis that predisposition to essential hypertension increases the risk of DN.

To elucidate whether the elevated V_{\max} of Na^+/H^+ exchange in DN was because of an increased NHE-1 density or an increased turnover per transporter site, we raised specific polyclonal antisera to the carboxyl terminus of NHE-1. This isoform appears to be the predominant one in lymphoblasts (15, 16), as NHE-2 and -3 mRNA transcripts are localized mainly to ion-transporting epithelia. Western blots of cell extracts enabled quantification of NHE-1 density in the different cell lines, and cell fractionation confirmed the localization of virtually all NHE-1 immunoreactivity to the surface plasma membrane, with no intracellular compartmentalization. The densities of NHE-1 immunoreactive bands were very similar among all three groups of cell lines, yielding an estimate of $\sim 22,000$ NHE-1 molecules per cell. Thus, the increased V_{\max} in DN could not be attributed to a higher number of transporter molecules per cell but clearly to an increased turnover number per site. This is consistent with our study in which we did not find differences in the expression of the NHE-1 gene in lymphocytes from patients with and without DN (Doria, A., J. H. Warram, and A. S. Krolewski, unpublished data). Post-translational processes such as N-linked glycosylation (33) and phosphorylation (21) have been demonstrated to alter Na^+/H^+ exchanger activity. However, even though there was size heterogeneity of NHE-1 in these cell lines, there were no significant differences in the distribution of the various molecular weight bands between the groups to suggest that altered activity was due to differences in N-linked glycosylation. It is possible that phosphorylation of NHE-1 plays a role in the increased turnover number of NHE-1 in DN, since we have demonstrated previously that staurosporine suppressed the V_{\max} of leukocytes of DN patients to levels reported in CON and DCON (7). This hypothesis remains to be tested directly in this cellular model of DN.

In conclusion, we have demonstrated that cultured lymphoblasts from patients with DN exhibit kinetic changes in the Na^+/H^+ antiport similar to those described in the HT phenotype (namely, a raised V_{\max} and a lowered Hill coefficient for internal H^+ binding). These changes in the antiport have persisted in cultured transformed cells, indicating the importance of genetic factors in determining this particular phenotype. The elevated V_{\max} in DN is not due to an increased NHE-1 density, but to an increased turnover number per transporter molecule, which is a further characteristic of this DN phenotype. The mechanism underlying this fundamental phenotypic change in DN is not known, but the immortalized lymphoblasts may provide a suitable model to define further the molecular nature of this ion transport abnormality.

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