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Leucocyte Na^+/H^+ antiport activity in Type 1 (insulin-dependent) diabetic patients with nephropathy

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Summary. The development of proteinuria in Type 1 (insulin-dependent) diabetic patients may depend on predisposition to essential hypertension in addition to poor glycaemic control. Previous work has shown increased leucocyte Na^+/H^+ antiport activity in essential hypertension and increased erythrocyte Li^+/Na^+ exchange in Type 1 diabetic patients with proteinuria. To test whether susceptibility to nephropathy in Type 1 diabetes was linked to abnormalities of leucocyte Na^+/H^+ antiport activity, we measured the intracellular pH and kinetics of the Na^+/H^+ antiport in 19 Type 1 diabetic subjects with, and 15 diabetic subjects without albuminuria and compared them to 25 matched normal control subjects. Intracellular pH (mean ± SD 7.59 ± 0.14) and maximal transport capacity of the antiport (V_{max} 87.7 ± 24.9 mmol · l^{-1} · min^{-1}) were higher in diabetic subjects with albuminuria compared to normotensive control subjects (pH 7.44 ± 0.09; V_{max} 55.6 ± 10.3 mmol · l^{-1} · min^{-1}; $p < 0.001$ for both), similar to the defect described in essential hypertension. These differences were not seen in diabetic subjects with normal urinary albumin/creatinine ratios (pH 7.46 ± 0.09; V_{max} 61.0 ± 13.6 mmol · l^{-1} · min^{-1}). Buffering characteristics of the leucocytes at different pH in the Type 1 diabetic subjects with albuminuria differed from normal control subjects and diabetic subjects with normal urinary albumin/creatinine ratios. We conclude that increased leucocyte Na^+/H^+ antiport activity, a known marker of essential hypertension, is usually associated with nephropathy in Type 1 diabetes.

Key words: Leucocytes, amiloride, Type 1 (insulin-dependent) diabetes mellitus, sodium.

Diabetic renal disease is one of the most common causes of endstage renal failure [1] but it is not known why only about one-third of all Type 1 (insulin-dependent) diabetic patients develop nephropathy [2]. Poor glycaemic control alone cannot account for the risk of developing this form of diabetic tissue damage [3]. Furthermore, long-term correction of hyperglycaemia with intensified insulin administration fails to stop the progression of renal damage in diabetic patients [4]. In contrast, treatment of hypertension can retard the decline in glomerular filtration rate in diabetic nephropathy [5]. Recent work has indicated that the risk of diabetic nephropathy in Type 1 diabetes is increased with parental history of hypertension, especially if associated with poor glycaemic control [6]. Moreover, the blood pressure of non-diabetic parents of diabetic patients with nephropathy is higher than that of parents of patients without nephropathy [7]. Thus, the risk of developing diabetic nephropathy may be linked to an inherited susceptibility to hypertension.

The erythrocyte Na^+/Li^+ countertransport activity has been shown to be raised in essential hypertension [8] and in Type 1 diabetic patients with nephropathy [6, 9]. Also, family studies have established that this countertransport is strongly genetically determined [10]. However, it is presently unknown what the natural substrate of this Na^+/Li^+ countertransport is.

Mahnensmith and Aronson have suggested that this countertransport may be one mode of operation of the ubiquitous Na^+/H^+ antiport [11]. Increased Na^+/H^+ antiport activity was subsequently documented in platelets [12] and leucocytes from hypertensive humans [13, 14] and in the spontaneously hypertensive rat [15, 16]. In this present study, we have determined the activity of the leucocyte Na^+/H^+ antiport in Type 1 diabetic subjects with and without albuminuria and in normotensive control subjects. Cellular buffering characteristics were also measured.

Subjects and methods

Nineteen Type 1 diabetic subjects with increased urinary loss of albumin (urinary albumin/creatinine ratio greater than 2 mg · $mmol^{-1}$, see below) were matched for age, body mass index and duration of diabetes with 15 patients without albuminuria. They were compared to 25 normotensive non-diabetic control subjects of similar age and body mass index. All subjects had normal renal function and were of European origin. None of the normal subjects were on any treatment including the oral contraceptive pill. The diabetic patients were

all on insulin therapy but omitted the morning dose on the day of the visit to the laboratory. Three of the albuminuric diabetic subjects were on beta blockers and/or nifedipine as antihypertensive treatment but had discontinued therapy for 2 weeks prior to the study. None of the other subjects were on beta blockers, calcium antagonists, angiotensin converting enzyme inhibitors, loop and thiazide diuretics, amiloride or digoxin. Informed consent was obtained from all subjects and the study was approved by the Oxford ethics committee.

Subjects collected a first morning urine specimen on three consecutive days and these specimens were analysed for urinary creatinine, sodium and potassium. Urinary albumin was determined by an immunoturbidimetric method [17] and albumin creatinine ratios computed in triplicate. Ratios above 2.0 mg albumin $\cdot$ mmol^{-1} creatinine were considered to be abnormally raised to maintain a sensitivity of detection above 95% [18]. Fourteen of the nineteen albuminuric diabetic patients had raised urinary albumin creatinine ratios for at least two years prior to the study, and the remaining five had albuminuria for at least three months. All subjects were free of urinary tract infection when studied as shown by culture of a midstream urine specimen. None of the subjects had clinical features of other auto-immune disease. Salt intake was unrestricted during the study.

Subjects arrived after an overnight fast and an i. v. cannula was inserted into an antecubital vein under local anaesthesia. After 30 min bed rest, supine blood pressure was measured in triplicate from the right arm using a Hawskley random zero sphygmomanometer and the average readings are reported. Then, 140 ml of blood was drawn for analysis of plasma creatinine and electrolytes, glucose by a glucose oxidase method (Beckman glucose analyser, Fullerton, Calif., USA), HbA_{1c} by agar gel electrophoresis (Corning Chemical, Palo Alto, Calif., USA), leucocyte intracellular pH and Na^+/H^+ antiport studies (see below). For the measurement of plasma free insulin, 5 ml of blood was immediately spun down in a refrigerated centrifuge (4 °C) and the plasma treated with polyethylene glycol to precipitate antibody bound insulin. The supernatant was frozen (20 °C) for later determination of insulin by RIA [19].

Leucocytes were separated by dextran sedimentation [20] and incubated at 37 °C for 30 min with the pH sensitive fluorescent dye bis (carboxyethyl) carboxyfluorescein acetoxymethyl ester (BCECF-AM), 10^{-5} mol$\cdot$l^{-1} final concentration. Cells were then washed three times in tissue culture medium 199 (containing 10% autologous serum by volume) and kept at room temperature for 30 min before any measurements were taken. Medium 199 was buffered with 15 mmol$\cdot$l^{-1} 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid (HEPES) and contained HCO_3^- derived from the autologous serum. All other buffers were nominally HCO_3^- free and this enabled the Na^+/H^+ antiport to be studied in isolation from other pH regulating systems, such as the Na^+ dependent and independent Cl^-/HCO_3^- exchangers. Also, the external Na^+ dependent H^+ efflux from acid loaded human leucocytes is largely inhibited by ethyl isopropyl amiloride (EIPA), a specific inhibitor of the Na^+/H^+ antiport. Furthermore, the addition of external HCO_3^- does not lead to any further increase in H^+ efflux, and this H^+ efflux is not sensitive to diisothiocyanato-stilbene disulphonate (DIDS), an inhibitor of Na^+ dependent and independent Cl^-/HCO_3^- exchange (unpublished observations). This means that acid loaded human leucocytes do not possess the Na^+ dependent Cl^-/HCO_3^- exchange, and the recovery of intracellular pH is dependent mainly on Na^+/H^+ exchange.

To measure resting intracellular pH, cells were washed once and resuspended in buffer containing (in mmol$\cdot$l^{-1}) NaCl 140, KCl 5, $CaCl_2$ 1.8, $MgSO_4$ 0.8, HEPES 15, glucose 5, pH adjusted to 7.4 (at 37 °C) with Tris base. The fluorescence of the intracellular BCECF was measured on a Perkin-Elmer luminescence spectrometer (LS-5B) equipped with a magnetic stirrer and thermostatically warmed cuvette holder. Excitation wavelengths were at 500 and 439 nm (slit width 2.5 nm) and emission wavelength set at 530 nm (slit width 10 nm). Intracellular pH readings were taken in duplicate. Fluorescence ratios (500/439 nm) were converted to pH units by constructing a 15 point calibration curve for every experiment in the pH range 5.9 to 7.8. This was done by measuring the 500/439 fluorescence ratio of cells in KCl/ionophore buffer containing (in mmol$\cdot$l^{-1}) KCl 140, $CaCl_2$ 1.8, $MgSO_4$ 0.8, HEPES 15, glucose 5, nigericin 2 (μmol$\cdot$l^{-1}), monensin 5 (μmol$\cdot$l^{-1}), pH 5.9 to 7.8. The monensin depletes the cells of Na^+ and the nigericin sets intracellular pH equal to extracellular pH [20]. Fluorescence ratios were converted to pH units using the following equation: –

$$F = A + B(10^{pH-pK}) / (1 + 10^{pH-pK})$$

where F is the fluorescence ratio (500/439 nm), A and B are constants and pK the pK of the intracellularly trapped BCECF. Typical values (mean ± SD) of these are A (1.354 ± 0.158), B (10.159 ± 0.708) and pK (7.061 ± 0.042).

Buffering power was determined by measuring the change in intracellular pH with the addition of extracellular 2–8 mmol$\cdot$l^{-1} $(NH_4)_2SO_4$ [20]. All readings were taken in duplicate.

In order to determine the kinetics of the Na^+/H^+ antiport for an individual, we used the two ionophores nigericin and monensin to clamp intracellular pH at values of 6.0, 6.3, 6.5, 6.8 and 7.1 by incubating cells in KCl/ionophore buffer (see above) at these different pHs for 5 min at 37 °C, and then scavenging off the ionophores with fatty-acid free bovine serum albumin (1 g$\cdot$l^{-1}) in KCl buffer (without the ionophores), as described in detail elsewhere [20]. The intracellular pH stays constant for as long as 20 min, in the absence of extracellular Na^+. On addition of 133 mmol$\cdot$l^{-1} NaCl (final concentration), a rapid change in intracellular pH results from exchange of intracellular H^+ with external Na^+, and about 95% of this flux is ethyl-isopropyl amiloride (EIPA) sensitive [20]. The initial rate of change of intracellular pH after addition of external Na^+ was computed for the first 20 s of the intracellular pH trace in the absence and presence of 10^{-5} mol$\cdot$l^{-1} EIPA. The EIPA sensitive rate of change of intracellular pH represents Na^+/H^+ antiport activity [20]. This measure (in U of pH U$\cdot$min^{-1}) when multiplied by the buffering power (in units of mmol$\cdot$pH^{-1}) yields proton efflux rate (expressed as mmol$\cdot$l$^{-1}\cdot$ min^{-1}). Buffering power was therefore determined at every pH studied, using the $(NH_4)_2SO_4$ technique [20]. All measurements were in duplicate.

Na^+/H^+ antiport kinetics were modelled using the Hill equation as follows:

$$J = J_{max}[H^+]^P / \{[H^+]^P + K^P\}$$

where J is the EIPA-sensitive H^+ efflux for a particular internal H^+ concentration $[H^+]$, J_{max} the maximum rate of H^+ efflux, K the equilibrium dissociation constant and P the Hill coefficient. Curves were fitted to the data using an algorithm based on the pattern search method [20] in order to determine the parameters J_{max}, K and P.

Leucocyte measurements were very reproducible and had the following coefficients of variation (within subject on different days): – intracellular pH (expressed as hydrogen ion concentration) 11.8%; J_{max} 7.3%. Part of the data from the 17 essential hypertensive subjects has been reported elsewhere [14, 21] and has been included only for comparative purposes.

Materials

Non-esterified-fatty acid free bovine serum albumin, nigericin, monensin and tissue culture medium 199 were from Sigma Chemical Co. Ltd, (Poole, Dorset, UK), and BCECF-AM from Calbiochem, (Cambridge Bioscience, Cambridge, UK). EIPA was a gift from Dr. A. Bearn, Merck, Sharp and Dohme, Rahway, NJ, USA.

Statistical analysis

Data were analysed using the Statistical Package for the Social Sciences (SPSS Inc., Chicago, Ill., USA). Multiple comparisons of variables between the different groups of subjects were performed using Duncan's test on untransformed data (reported as mean ± SD

Table 1. Clinical characteristics of the subjects in the study. Means and SD are reported and ranges are within parentheses [ ]. No significant differences between groups were found

	Type 1 diabetic subjects		Normotensive control subjects
	Albuminuria	No albuminuria	
Number (male)	19 (16)	15 (7)	25 (15)
Body Mass Index ($kg \cdot m^{-2}$)	23.7 ± 2.8 [17.5 – 28.9]	23.4 ± 2.3 [19.9 – 27.8]	24.1 ± 4.5 [18.0 – 37.0]
Age (years)	33.3 ± 11.2 [20.0 – 58.0]	37.9 ± 12.5 [23.0 – 64.0]	40.7 ± 15.2 [18.0 – 68.0]
Systolic blood pressure (mm Hg)	129.2 ± 18.2 [103 – 171]	126.9 ± 12.4 [110 – 147]	122.7 ± 11.1 [107 – 145]
Diastolic blood pressure (mm Hg)	78.5 ± 11.6 [61 – 110]	72.8 ± 5.9 [63 – 82]	78.1 ± 7.1 [63 – 89]
Duration of diabetes (years)	20.6 ± 8.7 [9 – 42]	23.1 ± 10.8 [8 – 42]	–
Insulin dose (U/day)	52.2 ± 16.3 [26 – 78]	47.4 ± 14.9 [26 – 72]	–

unless otherwise stated) or on log transformed data (reported as geometric mean [geometric mean + 1 SD]) if parameters did not show a Gaussian distribution. Only the plasma free insulin and urinary albumin/creatinine ratios were log transformed for analysis. Multivariate analysis of variance (ANOVA) was performed on the intracellular buffering power data at different intracellular pH in the three different groups of subjects (diabetic patients with and without albuminuria and normal control subjects). A 2 factor repeated measures design for ANOVA was used with 1 (group) between- and 1 (intracellular pH) within-subject factor. Pearson correlation coefficients were also calculated. *P* values quoted are 2-tailed.

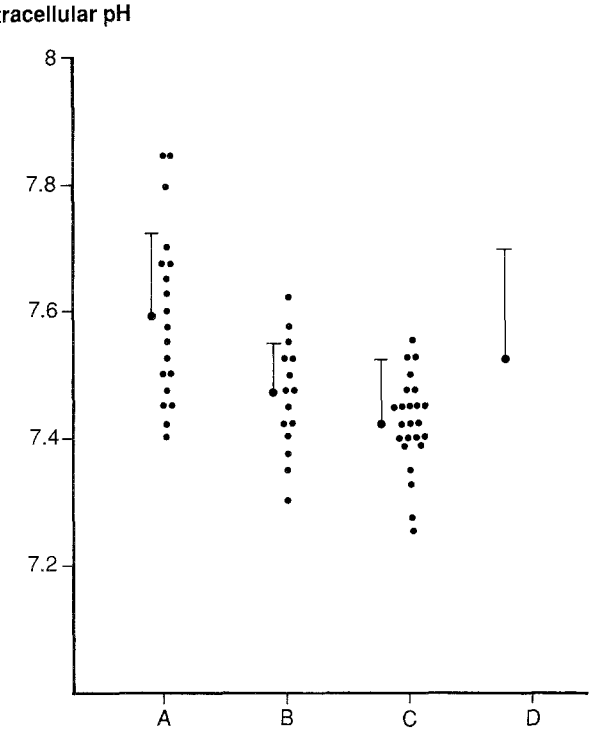

Fig. 1. Intracellular pH in diabetic subjects with (A) and without albuminuria (B), control subjects (C) and hypertensive patients (D). The data from the hypertensive subjects has been reported in part elsewhere [14, 21]. Means ± SD are plotted

Results

Table 1 shows that the Type 1 diabetic patients with and without abnormal albumin/creatinine ratios and the normal control subjects were well matched for age and body mass index. Systolic blood pressure was slightly higher in the group with albuminuria compared to normal control subjects but this difference did not reach conventional levels of statistical significance ($p = 0.1$). There was a predominance of males in the group with increased urinary albumin/creatinine ratios. The two groups of diabetic patients were well matched for duration of diabetes and daily insulin dosage. Nine diabetic patients in the group with albuminuria had proliferative retinopathy and four had background retinopathy. Of these diabetic patients without albuminuria, three had proliferative retinopathy and five had background retinopathy.

All subjects studied had normal and comparable plasma creatinine levels (Table 2). The fasting plasma free insulin levels were not different in the three groups of subjects, although fasting glucose and HbA_{1c} levels were significantly higher in both diabetic groups compared to the normal control subjects (Table 2, $p < 0.0001$ for both by ANOVA). However, the two diabetic groups had similar glycaemic control. Albumin/creatinine ratios were by definition significantly higher in the albuminuric group ($p < 0.0001$ by ANOVA), and the diabetic subjects without albuminuria had albumin/creatinine ratios which did not differ from the normal control subjects.

Leucocyte pH measurements showed higher resting values in the diabetic group with albuminuria when compared to both normal control subjects and diabetic patients without albuminuria ($p < 0.001$ by ANOVA; Table 3, Fig. 1). This diabetic group also had a slightly lower buffering power ($p < 0.005$ by ANOVA; Table 3). The Na^+/H^+ antiport kinetics determined by intracellular

Table 2. Fasting plasma and urine measurements in the two groups of diabetic and the normal control subjects. Means ± SD (for data showing a Gaussian distribution) or geometric means {geometric mean + 1 SD} (for plasma insulin and urinary albumin/creatinine ratios which were normalised by log transformation before analysis) are reported. Ranges are in parentheses [ ]

	Type 1 diabetic subjects		Normotensive control subjects
	Albuminuria	No albuminuria	
Fasting plasma glucose ($mmol \cdot l^{-1}$)	15.1 ± 5.2[a] [5.5 – 24.4]	12.5 ± 4.2[a] [5.5 – 18.9]	5.1 ± 0.4 [4.3 – 5.8]
Fasting plasma free insulin ($mU \cdot l^{-1}$)	7.9 {16.7} [1.6 – 22.2]	7.9 {11.7} [4.4 – 14.6]	7.8 {14.7} [2.1 – 39.3]
Plasma creatinine ($\mu mol \cdot l^{-1}$)	90.6 ± 14.6 [65 – 122]	78.7 ± 10.2 [65 – 99]	82.7 ± 11.4 [60 – 108]
HbA_{1c} (%)	9.96 ± 1.84[a] [7.2 – 12.6]	9.52 ± 2.16[a] [6.1 – 14.1]	6.01 ± 0.56 [4.8 – 7.0]
Urine albumin: creatinine ratio	11.4 {41.1}[a] [2.6 – 159.3]	0.4 {1.1}[b] [0.1 – 1.8]	0.3 {0.6} [0.1 – 1.5]

[a] $p < 0.001$ compared to normotensive control subjects (Duncan's test)

[b] $p < 0.001$ compared to Type 1 diabetic subjects with albuminuria

Table 3. Leucocyte intracellular pH and Na^+/H^+ antiport measurements in diabetic subjects with and without albuminuria, normotensive control subjects and hypertensive patients. The data from 17 hypertensive patients has been presented in part previously [14, 21]. Means ± SD are reported and ranges are in parentheses [ ]

	Type 1 diabetic subjects		Non-diabetic control subjects	
	Albuminuria	No albuminuria	Normotensive	Hypertensive
n	19	15	25	17
Resting intracellular pH	7.59[c] ± 0.14 [7.40 – 7.85]	7.46[d] ± 0.09 [7.29 – 7.62]	7.44 ± 0.09 [7.25 – 7.69]	7.53[a] ± 0.18 [7.26 – 7.95]
Intracellular buffering power ($mmol \cdot l^{-1} \cdot pH\ unit^{-1}$)	10.7[a] ± 7.0 [3.1 – 25.8]	16.1[d] ± 4.1 [10.0 – 25.8]	14.4 ± 4.8 [7.9 – 25.6]	10.4[a] ± 4.8 [3.0 – 18.0]
Maximal EIPA-sensitive H^+ efflux ($mmol \cdot l^{-1} \cdot min^{-1}$)	87.7[c] ± 24.9 [53.6 – 139.9]	61.0[e] ± 13.6 [38.0 – 92.4]	55.6 ± 10.3 [36.0 – 71.6]	74.6[b] ± 24.3 [36.4 – 123.3]
K_m (Dissociation constant) (pH units)	6.42[c] ± 0.14 [6.08 – 6.64]	6.67[e] ± 0.19 [6.36 – 7.00]	6.59 ± 0.15 [6.36 – 6.99]	6.53 ± 0.16 [6.22 – 6.76]
Hill coefficient	1.93 ± 0.44 [1.21 – 2.85]	1.67 ± 0.45 [1.08 – 2.63]	1.97 ± 0.89 [1.03 – 5.59]	1.87 ± 0.45 [1.04 – 2.68]

[a] $p < 0.05$, [b] $p < 0.01$, [c] $p < 0.001$ compared to normotensive control subjects using Duncan's test; [d] $p < 0.01$, [e] $p < 0.001$ compared to diabetic subjects with albuminuria using Duncan's test

pH clamping showed marked differences in the maximal transport capacity in the diabetic group with albuminuria compared to diabetic patients without albuminuria and normal control subjects ($p < 0.00005$ by ANOVA; Table 3, Fig. 2 and 3). The K_m of the antiport for intracellular H^+ ions was also different in this group, showing a reduced affinity for H^+ ($p < 0.0002$ by ANOVA; Table 3, Fig. 2). The Hill coefficients for both diabetic groups and the normal control subjects were similar. The diabetic subjects without albuminuria had intracellular pH, buffering power and Na^+/H^+ antiport kinetics very similar to those of normal control subjects. These differences between groups were not due to differences in the differential leucocyte counts (diabetic patients with albuminuria, polymorphonuclear leucocytes PMN 69 ± 11%, mononuclear leucocytes MNL 31 ± 10%; diabetic patients with

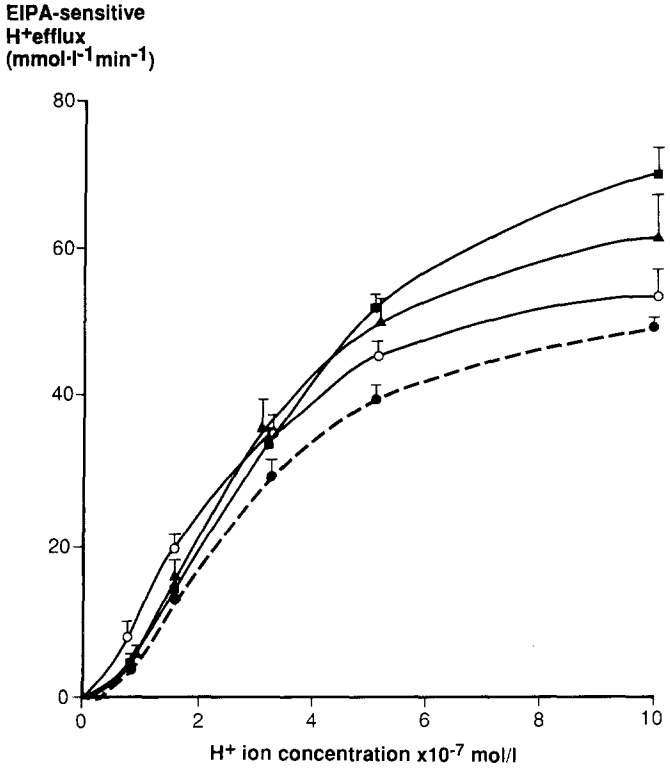

Fig. 2. The dependence of the Na^+/H^+ antiport activity on intracellular H^+ ion concentration. Na^+/H^+ antiport activity is defined as the ethyl isopropyl amiloride (EIPA) sensitive H^+ efflux. Means ± SEM are plotted for clarity. ● control subjects; ○ diabetic patients without albuminuria; ■ diabetic patients with albuminuria; ▲ hypertensive patients

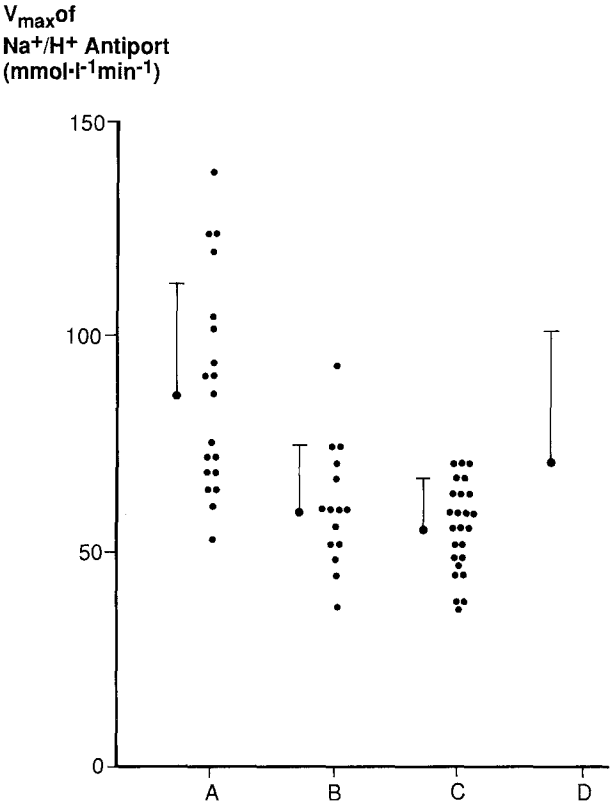

Fig. 3. The maximal transport capacity (V_{max}) of the Na^+/H^+ antiport in diabetic subjects with (A) and without (B) albuminuria, normotensive control subjects (C) and hypertensive patients (D). Means ± SD are plotted

Table 4. Leucocyte intracellular pH and Na^+/H^+ antiport measurements in male diabetic subjects with and without albuminuria and normotensive control subjects. Means ± SD are reported with ranges in parentheses [ ]

	Type 1 diabetic subjects		Non-diabetic control subjects
	Albuminuria	No albuminuria	
Resting intracellular pH	7.59[b] ± 0.13 [7.40 – 7.85]	7.49 ± 0.07 [7.42 – 7.62]	7.44 ± 0.08 [7.25 – 7.56]
Intracellular buffering power ($mmol \cdot l^{-1} \cdot pH\ unit^{-1}$)	11.1 ± 6.9 [3.1 – 25.8]	15.0 ± 2.7 [12.1 – 18.5]	14.7 ± 5.9 [7.9 – 25.6]
Maximal EIPA-sensitive H^+ efflux ($mmol \cdot l^{-1} \cdot min^{-1}$)	86.3[c] ± 24.9 [53.6 – 139.9]	53.7[e] ± 11.1 [38.0 – 72.5]	56.1 ± 11.5 [36.0 – 71.6]
K_m (Dissociation constant) (pH units)	6.43[a] ± 0.15 [6.08 – 6.64]	6.76[d] ± 0.21 [6.36 – 7.00]	6.59 ± 0.17 [6.36 – 6.99]
Hill coefficient	1.84 ± 0.35 [1.21 – 2.67]	1.43 ± 0.22 [1.08 – 1.77]	1.85 ± 0.13 [1.03 – 2.68]

[a] $p < 0.05$, [b] $p < 0.001$, [c] $p < 0.0001$ compared to normotensive control subjects using Duncan's test; [d] $p < 0.01$, [e] $p < 0.001$ compared to diabetic subjects with albuminuria using Duncan's test

Table 5. The intrinsic buffering power of leucocytes at different intracellular pH in the diabetic and normal control subjects. Buffering power is expressed in $mmol \cdot l^{-1} \cdot pH^{-1}$ and reported as means ± SD with ranges in parentheses [ ]

	Type 1 diabetic subjects		Non-diabetic control subjects
	Albuminuria	No albuminuria	
Intracellular pH			
7.1	6.97 ± 3.87 [2.49 – 17.97]	11.89 ± 4.63 [2.00 – 18.38]	13.52 ± 5.86 [3.66 – 28.57]
6.8	12.04 ± 5.09 [5.52 – 26.57]	15.21 ± 4.77 [5.33 – 23.05]	15.17 ± 5.19 [6.43 – 28.90]
6.5	25.99 ± 5.10 [18.26 – 39.29]	26.74 ± 6.57 [13.46 – 38.17]	23.71 ± 6.11 [12.93 – 41.22]
6.3	39.62 ± 9.41 [24.22 – 57.42]	35.77 ± 10.15 [25.00 – 62.78]	29.89 ± 6.49 [18.79 – 42.22]
6.0	45.17 ± 13.57 [30.66 – 77.51]	41.09 ± 8.95 [30.13 – 67.49]	37.85 ± 6.57 [22.69 – 48.99]

normoalbuminuria, PMN 67 ± 6%, MNL 33 ± 6%; normal subjects, PMN 66 ± 6%, MNL 34 ± 6%).

The diabetic subjects with albuminuria had values of the maximal transport capacity of the Na^+/H^+ antiport very similar to those we have previously reported for essential hypertensive patients elsewhere [21] (Table 3, Fig. 2 and 3). As previously reported, the non-diabetic hypertensive group showed an increased intracellular pH, reduced intracellular buffering power at resting intracellular pH and an increased maximal transport capacity of the Na^+/H^+ antiport [21].

The males from the two diabetic groups and the normal control subjects were analysed separately to examine any effect of sex. Table 4 shows that none of the above conclusions are altered. The diabetic subjects with albuminuria still had higher intracellular pH ($p < 0.002$ by ANOVA), higher maximal transport capacities of the Na^+/H^+ antiport ($p < 0.0001$) and lower affinity for intracellular H^+ ions ($p < 0.002$). There was no significant difference in Na^+/H^+ antiport activity between sexes in the normal control subjects. There were no significant correlations between the maximal transport capacity of the Na^+/H^+ antiport and plasma insulin, glucose, HbA_{1c}, body mass index or systolic blood pressure in both the diabetic groups and the normal control subjects. This parameter of antiport activity was only correlated to the diastolic blood pressure in diabetic subjects without albuminuria ($r = 0.54$, $p < 0.04$) and in both diabetic groups combined ($r = 0.46$, $p < 0.006$).

Table 5 shows the intracellular intrinsic buffering power of leucocytes from the two diabetic groups and the normal control group, measured at different intracellular pH. Multivariate analysis of variance showed a significant relationship of intracellular pH and buffering power in the different groups of subjects ($p < 0.001$).

Discussion

Two previous studies of nephropathy in insulin-dependent diabetes have shown that erythrocyte Li^+/Na^+ countertransport is increased [6, 9]. This membrane transport anomaly was initially described in essential hypertension [8] and shown to be primarily determined by genetic influences [10]. Thus, diabetic nephropathy may develop in diabetic patients who are also susceptible to essential hypertension [6, 9]. It has been suggested that this exchange may be a mode of operation of the Na^+/H^+ antiport [11]. However, the anucleate erythrocyte does not exhibit Na^+/H^+ exchange at rest [22] and thus is not suitable for measurements of Na^+/H^+ antiport activity. Membrane vesicles from mesenteric arteries do show Li^+/Na^+ exchange and Na^+/H^+ exchange, and both are inhibited to the same degree by ethyl isopropyl amiloride and phloretin [23]. Furthermore, the two modes of transport are not additive, and hence may occur via the same mechanism [23].

Previous work on leucocytes had indicated an increased Na^+/H^+ antiport activity in human essential hypertension [13, 14, 21] and in a rat model of genetic hypertension [16]. The present study demonstrated that the leucocytes from Type 1 diabetic patients with albuminuria were more alkaline intracellularly than those cells from diabetic patients with normoalbuminuria or normal control subjects. Furthermore, the same leucocytes had an increased Na^+/H^+ antiport maximal transport capacity. As all experiments were done in HCO_3^- free media, no Cl^-/HCO^-_3 exchange could have been responsible for any of the changes in H^+ flux measured. However, the changes in resting intracellular pH in vivo may be corrected by increased Cl^-/HCO_3^- exchange [24]. The net result could be intracellular overload with both Na^+ and Cl^-.

Diabetic subjects without albuminuria had intracellular pH and Na^+/H^+ antiport kinetics similar to the normal control subjects. However, the degree of glycaemic control in both groups of diabetic patients did not differ. The

changes in Na^+/H^+ antiport activity are therefore independent of glycaemic control. Blood pressure in the group with albuminuria was no different from that in normal control subjects. This may be because the numbers of subjects were too small to detect such a difference. Also, three of the albuminuric subjects were on anti-hypertensive treatment but had their therapy withdrawn for only two weeks, which may not be long enough for hypertension to be manifest. A further two albuminuric subjects have subsequently developed hypertension and it is possible that the blood pressure will rise in others from the group with albuminuria as the nephropathy progresses. We analysed the data separately for the male subjects only, and the conclusion that leucocyte intracellular pH was raised and the maximal transport capacity of the Na^+/H^+ antiport elevated remained unaltered. The incidence of diabetic nephropathy is higher in males [25] though the reason is unclear.

The intrinsic buffering power of leucocytes from diabetic patients with albuminuria also differed from that of normal control subjects. Buffering is not dependent only on the composition of the cytosol, but also on H^+ fluxes into and out of intracellular organelles [26]. The transporters that affect H^+ fluxes on such organelles have been only partially characterised [26], and it is possible that such pH regulating systems are different in the diabetic patients with albuminuria, in the same way that the plasma membrane pH regulating transporter, the Na^+/H^+ antiport, is more active in this group of patients.

It is not known how changes in Na^+/H^+ antiport activity could predispose to systemic hypertension. If the findings on leucocytes are also valid for vascular smooth muscle cells, two possible explanations may be considered. One is that the changes in intracellular ionic concentrations are directly responsible for the changes in vascular tone. For example, the increased Na^+/H^+ antiport exchange could lead to raised intracellular Na^+, hence raised intracellular Ca^{2+} by increased Na^+/Ca^{2+} exchange [27, 28]. Also, intracellular alkalinisation can directly enhance vascular contractility [29] by increased Ca^{2+}/calmodulin interaction [30] or increased generation of diacylglycerol in angiotensin-stimulated smooth muscle cells [31]. The second possibility is that the raised Na^+/H^+ antiport activity could be an exaggerated response to growth factor stimulation, which if present on vascular smooth muscle cells, could lead to vascular medial thickening [32]. This increased wall to lumen ratio could itself sustain hypertension [33]. Vascular agonists such as angiotensin II and catecholamines and growth factors such as epidermal growth factor and platelet derived growth factor could not only stimulate Na^+/H^+ antiport activity but also act as trophic stimuli for vascular smooth muscle cells [34, 35]. Further support for this notion comes from the increased smooth muscle cellular proliferation when cells are isolated from blood vessels of spontaneously hypertensive rats [37] and increased thymidine incorporation in blood vessels in vivo even in the pre-hypertensive stage [38].

Diabetic subjects who subsequently develop nephropathy have severe mesangial expansion with a progressive decline in glomerular filtration rate as the filtration surface area is gradually reduced [39]. It is uncertain whether the membrane transport defect we have described is a marker for the tendency for mesangial expansion to occur, for only 30% of Type 1 diabetic patients develop nephropathy.

In conclusion, leucocyte Na^+/H^+ antiport activity is increased in Type 1 diabetic patients with nephropathy irrespective of blood glucose or pressure levels. Such changes are similar to those described for essential hypertension. Further studies should determine the usefulness of this abnormality as a marker for development of renal impairment in diabetes, and may facilitate therapeutic intervention on blood pressure or glycaemic control before irreversible renal damage is initiated. The recent cloning of the Na^+/H^+ antiport gene may now allow tests to decide whether the abnormalities described above are due to alterations in the structure or the expression of this gene [40].

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