Leucocyte sodium efflux and electrolyte content in insulin treated diabetes

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Summary. Leucocyte sodium efflux and sodium content were studied in 41 insulin treated diabetic patients and compared to 41 age, body mass index and blood pressure matched non-diabetic control subjects. Fasting leucocyte ^{22}Na ouabain-sensitive efflux rate constants were lower in diabetic patients (median [range] 2.30 [1.04–3.73] versus 2.45 [1.57–3.95] h^{-1} , $p < 0.04$) suggesting a reduced sodium pump activity. The ^{22}Na ouabain-insensitive efflux rate constant which reflects passive sodium efflux was raised in insulin treated diabetes

(0.92 [0.42–1.73] versus 0.79 [0.28–1.49] h^{-1} , $p < 0.01$). Leucocyte sodium content was raised in the diabetic patients (47.7 [26.9–93.4] versus 26.5 [15.9–67.7] mmol/kg , $p < 0.0001$). Abnormal cellular sodium handling could lead to hypertension or other complications in diabetes.

Key words: Diabetes, leucocyte, sodium, adenosine-tri-phosphatase.

Type 1 (insulin-dependent) and Type 2 (non-insulin-dependent) diabetes are associated with a higher prevalence of hypertension [1, 2] and this may be one factor associated with the development of diabetic nephropathy [3, 4], a major cause of mortality in such patients. However, the mechanisms leading to the development of hypertension in diabetes are at present unclear, although some evidence for involvement of the catecholamine and renin-angiotensin systems exists [1], and genetic factors may also play a part [5]. One other unexplored possibility is the role of altered cellular sodium handling in diabetes.

Many cellular sodium transport abnormalities have been described in essential hypertension [6], including reduced leucocyte sodium efflux via the sodium pump [7] and increased erythrocyte Li^+/Na^+ exchange [8]. The defects in leucocyte Na^+ handling may explain the raised cellular Na^+ content in hypertension [7]. Also, such defects in leucocyte sodium pump activity may be genetically determined and exist even before the onset of hypertension [9, 10]. In Type 1 diabetes, erythrocyte $\text{Na}^+-\text{K}^+-\text{ATPase}$ activity is reduced [11], but there is no published work that we know of on human nucleated cells. Human leucocytes have been used as a model of vascular smooth muscle, the sodium pump activity of both being correlated [12]. We therefore studied leucocyte Na^+ efflux and intracellular electrolytes in insulin treated diabetic patients and

compared them to a group of age, body mass index and blood pressure matched non-diabetic control subjects.

Subjects and methods

All chemicals were Analar. ^{22}Na was from Amersham International (Aylesbury, Bucks., UK), TC199 (tissue culture fluid) from Wellcome Diagnostics Dartford, Kent, UK, and ouabain from the Sigma Chemical Company Poole, Dorset, UK.

Subjects

Forty-one insulin treated diabetic patients from the diabetic clinic (diastolic blood pressure < 95 mm Hg) and 41 age, body mass index and blood pressure matched non-diabetic subjects from local companies were studied after an overnight fast. All patients gave informed consent and the study was approved by the local ethics committee.

On arrival at 09.00 hours, an intravenous cannula was inserted and subjects rested supine for 30 min. Resting blood pressures were measured on a random zero sphygmomanometer by one observer, and the average of 3 values were reported. Eighty ml of blood was taken for plasma glucose, insulin, glycated haemoglobin and leucocyte sodium efflux and electrolyte studies. Subjects were excluded if they were on diuretics, beta-blockers, calcium antagonists, angiotensin-converting enzyme antagonists, oral contraceptives or anti-depressants, if they had had any infection within the previous month, or if their body weight had not been stable (± 3 kg) for the last

6 months. Also, all diabetic patients with hypertension, microalbuminuria, frank proteinuria or renal failure were excluded from the study. The diabetic patients omitted their human insulin on the day of the study. All subjects had normal plasma creatinine and electrolyte concentrations, and had no other endocrine disorder.

Leucocyte electrolyte content and efflux studies

Leucocytes were isolated from blood by dextran sedimentation as previously described [13]. 40 ml of blood (anticoagulated with preservative free sodium heparin 200 U) was mixed with 10 ml dextran (60 g/l TC199). After allowing the erythrocytes to settle for 30 min, the leucocyte-rich supernatant was carefully aspirated off and centrifuged at room temperature for 5 min at 160 g. Residual erythrocytes were lysed by hypo-osmotic shock. The leucocytes were washed once with TC199, and then half of the leucocytes were used for ^{22}Na efflux studies. Briefly, leucocytes were loaded with ^{22}Na in TC199 (final specific activity 1110 MBq/l) at 37 °C for 30 min. After centrifugation and one wash with TC199 at room temperature to remove most of the extracellular ^{22}Na , the efflux of ^{22}Na was measured into prewarmed (37 °C) TC199 over 20 min in the presence and absence of ouabain, 10^{-4} mol/l. Aliquots of cell suspension were pipetted at 4-min intervals into precooled Eppendorf tubes and spun down immediately. The radioactivity was measured in cell pellets and supernatants on a gamma counter. Efflux rate constants (ERC) were calculated from the slope of the regression of loge cellular radioactivity against time and expressed as h^{-1} . All ERC determinations had regression coefficients of greater than 0.99. The ouabain-sensitive ERC was defined as the difference between total and ouabain-insensitive ERC and reflects the activity of the sodium pump. The within-individual coefficient of variation for the ouabain-sensitive ERC was 7%, and that for the ouabain-insensitive ERC was 13%.

Intracellular electrolytes were measured in the other half of the leucocyte pellet. These were determined in fasting subjects as the level of intracellular Na^+ rises post-prandially [13]. The leucocytes were resuspended in TC199 at 37 °C for 30 min so that intracellular Na^+ , which tends to rise if cells are kept at room temperature, falls to a stable level. Thus, this half of the leucocyte pellet was treated identically and concurrently to that half of the pellet loaded with ^{22}Na for efflux studies, i.e. both had a period of incubation for 30 min in TC199 at 37 °C. This ensured that electrolyte composition in leucocyte pellets was similar for both the ^{22}Na efflux and intracellular electrolyte studies. After this period of incubation, the leucocytes were then spun down and washed three times with cold (4 °C) isotonic magnesium chloride buffer (MgCl_2 , 95 mmol/l, Tris base 5 mmol/l, pH 7.4 with HCl). There is minimal loss of cellular Na^+ with this washing procedure [13]. Pellets were then dried in an oven at 80 °C for 24 h before determining the dry weight to the nearest microgram. The residue was then dissolved in 1 mol/l nitric acid, and Na^+ and K^+ measured by flame photometry. The intracellular electrolytes are expressed as mmol/kg dry weight. The within-individual coefficient of variation for intracellular Na^+ was 11% and that for K^+ was 9%.

Other assays

Plasma glucose was measured by a glucose oxidase method, and glycated haemoglobin by agar gel electrophoresis [14].

Statistical analysis

All results are expressed as median [range]. The Mann-Whitney U test was used to compare the plasma and cellular measurements. Spearman correlation coefficients are reported.

Results

The insulin-treated diabetic patients and the non-diabetic control subjects were well matched for age, body mass index and both systolic and diastolic blood pressure (Table 1). The duration of diagnosed diabetes in the patients ranged from 6 months to 40 years. Although the leucocyte total ^{22}Na ERC did not differ in the 2 groups (diabetic subjects 3.18 [1.83–5.46] versus non-diabetic subjects 3.17 [2.13–4.79] h^{-1} , $p = \text{NS}$), the ouabain-sensitive ERC was lower in diabetic patients (diabetic patients 2.30 [1.04–3.73] vs control subjects, 2.45 [1.57–3.95] h^{-1} , Fig. 1, $p < 0.04$) and the ouabain-insensitive ERC was higher (diabetic patients, 0.92 [0.42–1.73] vs control subjects, 0.79 [0.28–1.49] h^{-1} , Fig. 1, $p < 0.01$). Thus, active Na^+ efflux per unit of intracellular Na^+ via the sodium pump was reduced, and passive Na^+ efflux was increased in the diabetic patients. Table 1 shows the very significantly increased leucocyte intracellular Na^+ and K^+ content in diabetic patients (intracellular Na^+ , diabetic patients 47.7 [26.9–93.4] vs control subjects, 26.5 [15.9–67.7] mmol/kg, $p < 0.0001$; intracellular K^+ , diabetic patients 402 [246–688] vs control subjects, 348 [202–514] mmol/kg, $p < 0.006$). The differences in Na^+ transport and electrolyte content were not due to differences in differential white cell count (diabetic patients, total

Table 1. General characteristics, plasma measurements and leucocyte intracellular electrolytes in insulin-treated diabetic patients and non-diabetic control subjects. Results are expressed as median [range]

	Insulin-treated diabetes	Non-diabetic
Number (male)	41 (25)	41 (29)
Age (years)	41 [22–69]	40 [16–65]
Body Mass Index (kg/m^2)	23.3 [19.4–30.2]	23.0 [19.1–29.7]
Systolic blood pressure (mm Hg)	123 [99–161]	120 [100–141]
Diastolic blood pressure (mm Hg)	75 [53–90]	79 [50–88]
Insulin dose (units/day)	47 [24–80]	–
Fasting plasma glucose (mmol/l)	13.0 [3.1–22.9]	5.1 [4.5–5.8]
Glycated haemoglobin (%)	10.1 [6.7–14.9]	–
<i>Leucocyte measurements</i>		
Intracellular Na^+ (mmol/kg)	47.7 [26.9–93.4] ^a	26.5 [15.9–67.7]
Intracellular K^+ (mmol/kg)	402 [246–688] ^a	348 [202–514]

^a $p < 0.001$ compared to non-diabetic control subjects

Fig. 1. Leucocyte ouabain-sensitive and insensitive ^{22}Na efflux rate constants in 41 non-diabetic subjects (●) and 41 age, body mass index and blood pressure matched diabetic patients (○). Medians are plotted

leucocyte count $6.1 [3.3-9.8] \times 10^9 \text{ l}^{-1}$, polymorphonuclear leucocytes 63 [36-77] %, lymphocytes 27 [17-52] %; control subjects, total leucocyte count $5.9 [3.5-9.5] \times 10^9 \text{ l}^{-1}$, polymorphonuclear leucocytes 57 [42-68] %, lymphocytes 31 [24-46] %).

In the diabetic patients, no significant correlations were found between the measurements of total, ouabain-insensitive and sensitive ^{22}Na efflux rate constants and intracellular electrolytes and the levels of glycated haemoglobin or fasting plasma glucose.

Discussion

Although a reduced Na^+ , K^+ -ATPase activity in the erythrocytes of Type 1 diabetic patients has been described [11], there has been no investigation of Na^+ transport mechanisms in human nucleated cells from diabetic patients. In this study, the leucocyte ^{22}Na ouabain-sensitive ERC, which reflects active Na^+ efflux via the sodium pump was reduced in the insulin treated diabetic patients. This would in turn lead to an elevation of intracellular Na^+ . These findings were not due to differences in differential white cell count, for these values were similar in the diabetic and control groups studied. Furthermore, we have shown previously that polymorphonuclear leucocytes and lymphocytes have similar electrolyte contents [13]. Finotti and Palatini [11] failed to demonstrate a raised intracellular Na^+ in Type 1 diabetes even with a reduced Na^+ , K^+ -ATPase activity, although they also found an abnormally high level of a plasma Na^+ , K^+ -ATPase stimulator in diabetes, which may have restored the erythrocyte sodium pump activity to normal in vivo. In addition, we have also found a raised intracel-

lular K^+ content in diabetes, but have no information on the K^+ permeability of the leucocytes. In animal models of diabetes, the onset of diabetes has been associated with a lowered Na^+ , K^+ -ATPase activity in nerve [15], dorsal root ganglia [16], heart [17] and muscle [18]. Further, Moore et al. found a raised soleus muscle intracellular Na^+ in rats with streptozotocin diabetes [18]. Our findings with the human leucocyte confirm these findings in other nucleated cells from animal tissue. However, the intracellular electrolyte content of cells incubated in tissue culture media in vitro may well differ from the intracellular composition of cells in contact with plasma in-vivo.

The ouabain-insensitive ERC was raised in insulin-treated diabetes. This passive Na^+ efflux is composed of Na^+/Na^+ exchange, loop diuretic-sensitive Na^+ , K^+ , Cl^- cotransport and membrane Na^+ permeability. We have not used the inhibitors phloretin or bumetanide to fractionate this passive efflux further. This increased passive efflux could be a compensatory response to intracellular Na^+ accumulation.

Increased erythrocyte Na^+ , K^+ , Cl^- cotransport has been described in essential hypertension [19]. Lithium-sodium exchange in erythrocytes, which may be a measure of Na^+/Na^+ exchange, is also raised in essential hypertension [8]. The lowered sodium pump activity in leucocytes from hypertensive subjects is also well documented [6, 7, 9]. The diabetic patients in this study had diastolic blood pressures less than 95 mm Hg, but whether the possession of such membrane transport defects could indicate a predisposition to the development of hypertension is not known at present. Such impairment of membrane transport systems has been observed in essential hypertension before the rise in blood pressure [10] and may be a genetic marker of susceptibility to the disease. As the parents of diabetic patients with nephropathy have higher blood pressures [5], and abnormally raised erythrocyte Li^+/Na^+ exchange has been found in diabetic nephropathy [20], it is uncertain to what extent the defects in leucocyte Na^+ transport may be markers of later development of hypertension or nephropathy.

A further consequence of intracellular Na^+ accumulation due to reduced active Na^+ efflux may be a lowering of the influx of metabolites which are dependent on the inward Na^+ gradient to drive them into the cell against their concentration gradient. Examples include amino-acids [21] and inositol [22], and there is evidence for impaired uptake of such substances in animal diabetic tissue [22, 23]. Whether a deficiency of such substances could lead to some of the complications of diabetes, like neuropathy, is unknown.

In conclusion, leucocytes from insulin-treated diabetic patients had a lowered ouabain-sensitive $^{22}\text{Na}^+$ efflux rate constant and a raised ouabain-insensitive ^{22}Na efflux rate constant. The intracellular

Na^+ content was raised, probably a consequence of reduced sodium pump activity. Such abnormalities in membrane Na^+ transport could exist even in the absence of the vascular or renal complications of diabetes, but whether they indicate susceptibility to complications such as nephropathy or hypertension is at present unknown.

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