Enhanced erythrocyte Na$^+$/H$^+$ exchange predicts diabetic nephropathy in patients with IDDM

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Summary Diabetic nephropathy develops in a subset of patients with an apparently hereditary predisposition. Microalbuminuria and elevated arterial pressure have been proposed as predictors of nephropathy but both appear when renal damage is impending. Enhanced sodium-hydrogen exchange in the cell membranes of diabetic patients is an early marker of diabetic nephropathy but its predictive value has not been assessed. In this study, sodium-hydrogen exchange was measured in erythrocytes as an initial velocity of amiloride-inhibited H$^+$ efflux (pH 6.35–6.45) into a Na$^+$-containing medium (pH 7.95–8.05) in 156 non-microalbuminuric insulin-treated diabetic patients (98 women, 58 men, age 33 ± 8 years, diabetes duration prior to enrollment 15 ± 4 years) during 8 years of follow-up. Enhanced erythrocyte sodium-hydrogen exchange predicted diabetic nephropathy alone and in association with a familial tendency to hypertension/nephropathy with 86 and 96 % sensitivity, and 80 % specificity. Thus, sodium-hydrogen exchange appears to detect a subset of diabetic patients prone to develop renal damage, in whom a more intensive treatment modality might be considered. [Diabetologia (1998) 41: 201–205]

Keywords Na + /H + exchange, IDDM, diabetic nephropathy, erythrocytes.

Diabetic nephropathy is limited to a subset of patients with long-standing poorly controlled diabetes and an apparent hereditary predisposition [1, 2]. To depict the subset of patients prone to renal damage, several markers, e.g. urinary excretion of very small amounts of albumin (microalbuminuria) [3] and elevation of arterial pressure [4], have been proposed. However, these symptoms appear when the renal damage is already impending [5].

Enhanced sodium-lithium countertransport and sodium-hydrogen exchange (NHE) found in blood cells and skin fibroblasts of diabetic patients prone to nephropathy [6–10] represent a very early (if not congenital) sign. Its accuracy in predicting diabetic nephropathy has not been established. In this study we have followed-up non-microalbuminuric patients with IDDM of at least a 10-year duration for 8 years after their first NHE evaluation.

Subjects and methods

Study group. We followed-up 156 patients (98 women, 58 men) aged 33 ± 8 years, with juvenile onset of insulin-dependent diabetes mellitus (diabetes duration prior to enrollment 15 ± 4 years) for 8 years (total diabetes duration 23 ± 3 years). The criteria for the patients' enrollment in the study were: duration of insulin treatment of at least 10 years and up to 25 years, absence of proteinuria/microalbuminuria, normal arterial blood pressure, normal serum urea, creatinine and electrolyte levels, and absence of clinically evident retinopathy at the time of enrollment. Anamnestic or clinical evidence of recurrent or chronic urinary infection was the single exclusion criterion both on enrollment and during the follow-up. Any

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Abbreviations: NHE, Sodium-hydrogen exchange; IDDM, insulin-dependent diabetes mellitus; MAP, mean arterial pressure.

first-to-second degree relatives treated for elevated blood pressure or renal disease were registered on the patients' personal records.

The diagnosis of diabetes was revised according to the international criteria [11]. The patients' blood pressure was measured as recommended by the Joint National Committee on Detection, Evaluation and Treatment of High Blood Pressure [12], diastolic blood pressure was defined by disappearance of the Korotkoff sounds phase V. Established hypertension was defined as the mean of three different blood pressure measurements over 160/95 mm Hg. Mean arterial pressure (MAP) was calculated as $1/3$ (Systolic BP + $2 \times$ diastolic BP). Elevated MAP was defined as more than 115 mm Hg, which corresponded approximately to a blood pressure of 160/95 mm Hg. Urinary albumin secretion was determined by a solid-phase fluoroimmunoassay [13]. Urinary excretion of albumin between 20 and 300 mg/24 h was defined as microalbuminuria. Creatinine levels of 135 μmol/l and more was defined as azotaemia. The daily insulin requirement was estimated as the mean insulin dose during the last 7 days.

During the annual examinations the patients underwent measurements of blood HbA_1, serum urea and creatinine, and urine protein/albumin (single urine sample per patient). The daily insulin requirements, hypoglycaemic episodes and ketoses during the previous year were noted. The patients who had developed overt proteinuria or azotaemia underwent ultrasound examination of the kidneys. The triade of enlarged kidneys, proteinuria and long progression of renal damage was considered typical of diabetic renal disease [14].

The study protocol was approved by the hospital ethical committee. Informed consent was obtained from each patient enrolled in the study.

Sodium-hydrogen exchange study. Sodium-hydrogen exchange (NHE) was determined as amiloride-dependent fraction of proton efflux from acidified erythrocytes into an alkaline sodium-containing medium [15, 16]. For this purpose, the venous blood was drawn into plastic tubes containing 50 IU heparin/ ml of blood and stored ice-cold for no more than 6 h. Before the blood sampling, the patients had fasted for at least 8 h. The erythrocytes were sedimented and washed three times with a medium containing phosphate buffer (pH 7.4).

Packed erythrocytes (100 μl) were incubated with a 1.9 ml of solution containing (mmol/l) 150 NaCl, 1 KCl, 1 $MgCl_2$ and 10 glucose (no buffer added) for 5 min at 37 °C. The pH of the cell suspension was adjusted to 6.35–6.45 by addition of HCl. The anion exchanger was inhibited by 200 μmol/l 4,4′-diisothiocyanostilbene-2,2′-disulphonic acid (DIDS; Sigma, St. Louis, Mo., USA). Then the pH was adjusted to 7.95–8.05 by rapid NaOH addition. The kinetics of H^+ efflux were registered by a pH meter (Radiometer; Copenhagen, Denmark). Each probe was performed again with 500 μmol/l amiloride (Sigma Chemical Co) added to the cell suspension after DIDS. NHE was defined as initial velocity of amiloride-inhibited proton efflux (v_{init}) under the above-mentioned conditions, and calculated as $(\Delta pH - \Delta pH_{amiloride})$ b/mt, where ΔpH is the initial rate of the medium acidification, in the absence and presence of amiloride, respectively, b is the buffer capacity of the medium, m is the erythrocyte volume, and t is the incubation time. The buffer capacity at pH 8.00 was estimated before each experiment by titration of 1.9 ml of the incubation medium with HCl and NaOH solutions (pH ranging from 6.00 to 8.00) and calculated as mmol H^+/unit pH. The buffer capacity varied from 0.3 to 0.8 μmol H^+/unit pH. NHE measurements were performed during the initial examination, and at 5 and 8 years of follow-up. The last NHE measurement was performed at pH 6.35–6.45 (v_{init}) and at pH 6.00–6.05 (V_{max}). The upper limit of the 'normal' NHE

levels was estimated as the mean for the control population + 2 SD, or 200 μmol $H^+ \cdot l$ cells$^{-1} \cdot$ min^{-1}. The control population comprised 200 subjects (100 men and 100 women) aged 40–60 (mean 31 ± 8) years, with no individual or familial history of hypertension, diabetes mellitus or renal disease [17].

Statistical analysis. All values are expressed as mean ± SEM. Significance was tested by using the comparative distribution of variables by chi-square test. Multiple logistic regression was used for computing standardized regression coefficients. *P* values less than 0.05 were considered statistically significant.

Results

Sodium-hydrogen exchange (NHE) was enhanced in erythrocytes of 63 (40.4 %) patients with IDDM, whereas the prevalence of enhanced NHE in the control population was 9.2 %. The IDDM patients with high and low/normal NHE had similar blood pressure levels and similar durations of diabetes (Table 1). The glycaemic control during follow-up was also similar in IDDM patients with enhanced and low/normal NHE, as were the daily insulin requirements. No correlation between NHE rate and daily need for insulin was observed in the study group. The range of hypoglycaemic events and ketoses did not differ between the patients with relatively high or relatively low NHE levels. However, the individuals with enhanced antiport activity had on average more relatives with hypertension and renal disease.

The antiporter velocity remained stable during the years of the follow-up in IDDM patients with and without nephropathy (Table 2).

During 23 ± 3 years of the total duration of diabetes, diabetic nephropathy (overt proteinuria and/or azotaemia) developed in 49 (31.4 %) of patients. The cumulative incidence of nephropathy (overt proteinuria and/or azotaemia) was significantly higher in IDDM patients with high NHE activity (Fig. 1). The consequences of diabetes in patients with increased and normal/low NHE are listed in Table 3. The elevated MAP appeared at 14.8 ± 2.5 years of diabetes duration, earlier than development of microalbuminuria (21.2 ± 1.4 years, $p = 0.03$).

Enhanced erythrocyte NHE was a good predictor of diabetic nephropathy, with sensitivity of 86 %, specificity of 80 % and accuracy of 82 %. Accuracy of the NHE measurements in predicting diabetic nephropathy increased to 86 % when the rate of NHE was accompanied by anamnestic evidence of hypertensive first-to-second degree relatives and/or elevated MAP levels, and/or familial tendency to nephropathy, with a sensitivity reaching 96 % in these cases.

Initial NHE velocity at pH 6.35–6.45 (v_{init}) correlated with the maximal NHE velocity (pH 6.00–6.05, V_{max}; $r = 0.78$, $p < 0.01$). The relative risk of nephropathy was 8.9 [95 % CI, 2.2–15.6] in diabetic patients with enhanced NHE V_{max}, and 30.1 [95 % CI, 25.1–

Table 1. The characteristics of the studied groups

Factors	High[a] NHE (n = 63)	Normal NHE (n = 93)	P values
Men	24	34	–
Women	39	59	–
Age (years)	32 ± 4	33 ± 7	NS
Blood pressure (mmHg)			
systolic	143 ± 11	141 ± 10	NS
diastolic	82 ± 4	84 ± 7	NS
Hypertensive relatives[b]	1.06 ± 0.2/patient	0.34 ± 0.1/patient	0.03
Relatives with renal disease	0.31 ± 0.2/patient	0.14 ± 0.1/patient	0.01
Diabetes duration prior to enrollment (years)	15 ± 6	14 ± 7	NS
HbA_1 (%)			
mean for 5 years before enrollment	11 ± 1	10 ± 1	NS
on enrollment	11 ± 2	11 ± 2	NS
at 8-year follow-up	10 ± 2	10 ± 1	NS
Insulin requirement on enrollment (IU/day)	64 ± 12	61 ± 7	NS
Hypoglycaemic events	5.2 ± 1.2/year	4.9 ± 2.0/year	NS
Ketosis	1.6 ± 1.0/year	1.7 ± 0.6/year	NS

Data are mean ± SEM
[a] High NHE $> 200\ \mu$mol $H^+ \cdot 1$ cells$^{-1} \cdot$ min^{-1} estimated as mean + SD during the population studies [W. Koren, data not shown];
[b] first-to-second degree relatives
NS, Non-significant

Table 2. Na^+/H^+ exchange in IDDM patients during 8 years of follow-up (total, $n = 156$)

	V_{init}			V_{max}
	At enrollment	After 5 years	After 8 years	At 8 years
NHE levels[a]	241 ± 12	240 ± 8	234 ± 12	312 ± 26

Data are mean ± SEM
[a] μmol $H^+ \cdot 1$ cells$^{-1} \cdot$ min^{-1}

35.0] in patients with high NHE V_{max} and positive family history of hypertension and/or renal disease.

Microalbuminuria was less sensitive but more specific in predicting the diabetic renal disease (sensitivity 32%, specificity 92%, accuracy 86%). The results of multiple regression analysis of other potential predictors of diabetic renal disease are presented in Table 4.

Discussion

Diabetic nephropathy is the most frequent cause of end-stage renal failure [18] and a leading cause of mortality resulting from insulin-treated diabetes [19]. Since its susceptibility does not exceed one third of IDDM patients and is exhausted over 15–17 years of diabetes [20], the hereditary predisposition to this type of microangiopathy seems evident.

Numerous observations propose an association between diabetic nephropathy and arterial hypertension in diabetic patients [21, 22] as well as parental hypertension and hypertension in first-to-second degree relatives [23] and enhanced erythrocyte Na^+/Li^+ countertransport [24], which is generally attributed to a mode of action of Na^+/Na^+ (Na^+/H^+) exchange.

Na^+/H^+ exchange (NHE) represents a protein which is expressed ubiquitously in plasma membranes of most studied human cells [25]. Its enhancement in essential hypertension is presumably explained by certain genetic factors [26]. The results of this study show that enhanced NHE in diabetic patients is the earliest sign of susceptibility to nephropathy, when compared to elevated MAP and microalbuminuria. NHE levels remain stable during the follow-up. There is a clear familial predisposition to hypertension and renal disease in patients with enhanced NHE. We therefore suggest that the NHE enhancement in diabetic patients prone to renal disease may be mediated by hereditary mechanisms which apparently overlap those involved in NHE stimulation in patients with essential hypertension.

Enhanced NHE has been found in 5–10% of the healthy population, and up to 50–60% of patients with essential hypertension [17]. In this study, its prevalence in IDDM has been estimated to be 40.4%, i.e. higher than the reported 56% prevalence of increased Na^+/Li^+ countertransport in microalbuminuric patients with IDDM [27]. The possible explanations for this discrepancy include: 1) different sensitivity of the assays used for their measurement; 2) different affinity of the exchanger to Na^+, H^+ and Li^+ at different pH levels (NHE measurement is carried out at pH ranging from 6.00 to 8.00, whereas Na^+/Li^+ countertransport is measured at physiological pH), and 3) geographic peculiarities of high NHE
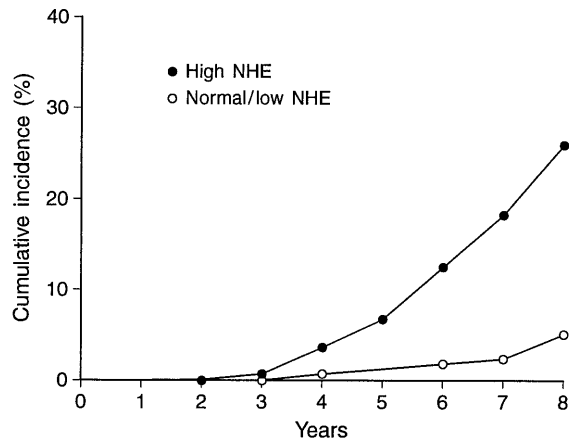
Fig. 1. Cumulative incidence rates of diabetic nephropathy (total duration of diabetes 23 ± 3 years) over 8 years of follow-up according to NHE activity (high[a] vs normal/low) [a]High NHE $> 200\,\mu mol\ H^+ \cdot l\ cells^{-1} \cdot min^{-1}$ estimated as mean + SD during the population studies

Table 3. Consequences of diabetes in patients with high[a] or normal Na^+/H^+ exchange

Diabetic consequences	High NHE ($n = 63$)	Low/normal NHE ($n = 93$)	P value
Elevated MAP	49 (78 %)	16 (17 %)	0.02
Established hypertension	31 (49 %)	11 (12 %)	0.03
Microalbuminuria	61 (97 %)	8 (9 %)	0.001
Overt proteinuria	29 (46 %)	9 (10 %)	0.03
Azotaemia	18 (29 %)	4 (4 %)	0.02

[a] High NHE $> 200\,\mu mol\ H^+ \cdot l\ cells^{-1} \cdot min^{-1}$ estimated as mean + 2 SD during the population studies [W. Koren, data not shown]

Table 4. Other possible predictors of diabetic nephropathy in IDDM patients (logistic regression analysis)

	Coefficient	Standard error	P value
Age	0.061	0.011	0.04
Systolic blood pressure	0.432	0.017	0.01
Diastolic blood pressure	0.612	0.022	0.001
Duration of diabetes	0.321	0.042	0.005
HbA$_1$ level	0.412	0.070	0.01
Insulin requirement	–0.212	0.051	0.08

and high Na^+/Li^+ countertransport distribution in the population.

The strict mechanism of NHE enhancement in IDDM patients remains obscure. In vitro the higher insulin concentrations have been shown to augment NHE [27]. However, our results deny a role for insulin in NHE stimulation in vivo. Indeed, no association between NHE and insulin requirement has been found in insulin-treated diabetic patients, and there was no NHE enhancement in individuals (with or without nephropathy) after the doses of insulin had been raised (data not shown).

High erythrocyte NHE appears to be a sensitive test to detect the subset of IDDM patients prone to diabetic renal disease. The rate of microalbuminuria, which is a less sensitive but a more specific test, maintains its potential in the follow-up and secondary prevention of diabetic nephropathy.

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