Abnormal *myo*Inositol Influx in Human Leucocytes in Diabetes but not Specifically in Diabetic Neuropathy

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Abnormal *myo*inositol metabolism has been implicated as a contributor to the development of diabetic neuropathy. Furthermore, in vitro glucose inhibits animal and human *myo*inositol transporters. To investigate whether *myo*inositol transport is abnormal in diabetic subjects with and without neuropathy, we used a triple-isotope technique to measure [^{14}C]*myo*inositol uptake in leucocytes from 23 insulin-dependent diabetic subjects and 13 matched nondiabetic subjects. All subjects with diabetes underwent neurophysiological studies, and subjects without neuropathy were compared with those with various degrees of neuropathy. The relationship between glycemia and flux was also studied. Diabetic subjects had similar intracellular and plasma *myo*inositol concentrations but had higher rates of uptake of *myo*inositol over the extracellular concentrations of *myo*inositol studied. Although the derived K_m, V_{max}, and passive components were not significantly different, the V_{max}:K_m ratio was significantly higher in diabetic subjects compared with nondiabetic subjects (0.25 [0.17–0.32] vs. 0.16 [0.13–0.19], respectively ($P = 0.006$). In diabetic subjects, the rate of *myo*inositol uptake correlated with HbA_{1c}, particularly at 3 μM extracellular *myo*inositol where active uptake was a high proportion of the total influx ($P < 0.005$). No difference in *myo*inositol uptake was found among diabetic subjects with various degrees of neuropathy. We conclude that although *myo*inositol transport is abnormal in diabetes, it is not specifically abnormal in diabetic neuropathy. Prolonged hyperglycemia is associated with higher *myo*inositol flux. *Diabetes* 41:760–65, 1992

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The initiating process for the development of the permanent structural abnormalities in diabetic neuropathy is unknown. The association with hyperglycemia has led to the suggestion that an increase in intracellular glucose flux results in accumulation of sorbitol via the polyol or aldose reductase pathway and to nerve damage (1). In the diabetic nerve of diabetic humans and animals, a raised intracellular sorbitol concentration has been shown, and in animal and postmortem human nerve this is associated with reduced levels of *myo*inositol (2–4), although no such decrease is found in sural nerve biopsies (5). Some have postulated that lower intracellular concentrations of *myo*inositol may contribute to the pathogenesis of neuropathy either by impairing Na^+-K^+ ATP activity (6) or by reducing production of phosphatidylinositol (PI) (a constituent of cell membranes) by the low-affinity enzyme PI synthase (7). Although it is known that *myo*inositol enters cells via a sodium-dependent glucose-inhibitable ouabain-sensitive transporter (8–11), the precise mechanism leading to the reduced intracellular *myo*inositol concentration in diabetic cells remains unknown.

We have previously characterised the *myo*inositol transporter in human leukocytes (8). Leukocyte *myo*inositol influx is dependent on extracellular sodium and a source of energy and is inhibited competitively by extracellular glucose and ouabain. These characteristics resemble the *myo*inositol influx properties, described by Greene and Latimer, in rabbit nerve (9). The K_m in leukocytes was found to be ~61 μM in 0 mM glucose (8), which is in the same relative concentration as the transporter in rabbit nerve (63 μM in 0 mM glucose). Thus, both leukocytes and nerve possess a high affinity and specific external sodium-dependent *myo*inositol uptake mechanism. We therefore used the leukocyte as an accessible model of *myo*inositol uptake to investigate the

changes in *myo*inositol balance in human diabetic subjects with and without diabetic neuropathy.

RESEARCH DESIGN AND METHODS

The studies were approved by the Central Oxford Research Ethics Committee, and all subjects gave informed consent. All notes from the Oxford diabetic outpatient clinic were reviewed. Patients treated with insulin within 1 mo of diagnosis, with diabetes for >18 yr and with onset while aged <35 yr or proven ketoacidosis (or a fasting C-peptide <0.06 nM), were considered to have insulin-dependent diabetes mellitus. Patients were invited to participate in the study if they had no proteinuria, were on no medication (including oral contraceptives) besides insulin (except thyroxine in 1 case); and without any other endocrine, psychiatric, or metabolic disorder. Nondiabetic control subjects, recruited via the laboratory staff, had fasting glucose levels <5 mM, were on no medication, and had no medical disorders.

No subjects had travelled abroad or had any infection in the previous 4 wk. All subjects fasted overnight, and, on arrival between 0800 and 1030, each had an intravenous cannula inserted into an antecubital vein. The subjects rested for 0.5 h, and blood pressure was measured on the other arm by one observer (D.S.) using a random zero sphygmomanometer. Blood (180 ml) was then taken for transporter studies, plasma and intracellular *myo*inositol assay, creatinine, glucose, and free insulin in all subjects and HbA_{1c} and C-peptide in diabetic subjects. C-peptide was measured by radioimmunoassay (CIS, High Wycombe, UK; within-batch coefficient of variation [CV] of 7.1%, between-batch CV of 10.7%, sensitivity of 0.05 ± 0.01 nM^{-1}), glucose by a glucose oxidase method (Beckman glucose analyzer, Fullerton, CA), and HbA_{1c} by agar gel electrophoresis (Corning, Palo Alto, CA). Free insulin was measured by double radioimmunoassay after treatment with polyethylene glycol (12).

Besides standard neurological history and examination, nerve conduction tests were performed at the Oxford University Department of Neurophysiology by a consultant neurophysiologist. All neurophysiological assessments were conducted using surface electrodes on the right side after subjects were made comfortable in a warm environment, with skin temperatures maintained above 30°C. Compound muscle action potential, distal motor, and F-wave latency were measured in the posterior tibial and ulnar nerves and amplitude and peak latency were measured in the sural, median, and ulnar nerves. Thermal thresholds were measured at the wrist and ankle. Normal ranges for electrophysiological measurements were defined as those described by Sethi and Brown (13). The neurophysiologist coded the severity of the neuropathy as mild if neurophysiological measurements were abnormal only in the feet and as moderate/severe if both the feet and hands were affected. These tests and the coding were performed without knowledge of the transport study results.

Transporter studies. Materials for *myo*inositol transport study came from the following sources: ^3H_2O, 2-[^{14}C]*myo*inositol, and $^{22}Na^+$ came from Amersham (Bucks, UK) and phloretin, ouabain, and dibutyl phthalate came from Sigma (Poole, Dorset, UK). TCI99 (tissue culture medium) came from Wellcome Diagnostics (Dartford, Kent, UK) and was adjusted with NaCl and KCl to the composition of (mM) Na^+ 140, K^+ 5.0. Other reagents were of the highest possible grade (BDH Chemicals, Poole, Dorset, UK). All solutions were at pH 7.4 at 37°C, as measured by a radiometer ION83, (Copenhagen) after pH adjustment with Tris base and weak hydrochloric acid (final osmolality 285 mOsm/kg, i.e., identical with TC199).

***myo*inositol uptake assay.** Leukocytes were separated by dextran sedimentation, washed once with TC199, resuspended in TC199 with 10% autologous serum, then incubated in a water bath at 37°C for 0.5 h as previously described (8). The suspension was divided into five equal aliquots and washed twice in incubation buffer consisting of 15 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid, 140 mM Na^+, 1.8 mM Ca^{2+}, 5 mM K^+, 0.8 mM Mg^{2+}, 1 mM glutamine, and either 0.003, 0.01, 0.05, 0.1, or 0.15 mM of *myo*inositol. Each aliquot was centrifuged, the supernatant was aspirated, and 200 μl of the same prewarmed incubation buffer was added to the cell pellet. The cells were gently resuspended, returned to the water bath for 1 min, and then the suspension was added to 20 μl of [^{14}C]*myo*inositol, making a final specific activity of ^{14}C of 84 kBq/ml of buffer.

Triplicate 30-μl aliquots were pipetted at 5 and 10 min after adding the labeled *myo*inositol, and each was placed into a chilled (4°C) stop solution containing 95 mM $MgCl_2$, 5 mM Tris, 7.4 mBq of ^{22}Na/ml (to calculate extracellular volume), 185 mBq of ^3H_2O/ml (to calculate total volume), 1 mM amiloride, and 1 mM phloretin (to prevent any ^{22}Na uptake by the cells). *myo*Inositol influx was stopped by the entry of the [^{14}C]*myo*inositol into the stop solution and by immediately centrifuging the cells through a 50 μl layer of dibutyl phthalate. The remaining cell suspensions were spun down and a 15 μl aliquot of supernatant taken from each.

The stop tubes were frozen at −70°C after 50-μl aliquots of supernatant were taken from each of the triplicates. The cell pellets then were isolated by cutting through the frozen oil. Aliquots of the supernatants and cell pellets were dissolved in 0.5 ml 10% sodium dodecyl sulfate, and 7 ml of scintillant was added. Activities were counted on a Beckman LS 5801 triple counter with appropriate quench corrections for all three isotopes (^{22}Na, ^{14}C, and ^{3}H).

Calculation of influx rate. The rate of [^{14}C]*myo*inositol influx was calculated by plotting the intracellular [^{14}C] concentration against time, then using a least-squares technique. The accumulation of intracellular [^{14}C]*myo*inositol was computed as follows:

$$\text{myoinositol in pellet (mM)} = \frac{\textit{dpm of intracellular} \text{ myoinositol in pellet}}{\text{dpm of intracellular } {}^3H_2O \text{ in pellet}}$$

$$\times \frac{^3H_2O \text{ per unit volume (dpm/L)}}{\text{spec activity } myo\text{inositol (dpm/mM)}}$$

i.e.,

$$[MI] = \frac{MI_p - [(Na_p \times MI_s)/Na_s]}{H_p - [(Na_p \times H_s)/Na_s]}$$

$$\times \frac{H_s}{(MI_{sa} \times V_m)}$$

where Na_p and Na_s, MI_p and MI_s and H_p and H_s are the ^{22}Na dpm, ^{14}C dpm, and ^3H dpm counts in the pellet and stop solution respectively, V_m is the volume of stop solution counted (in l) and MI_{sa} is the final specific activity of [^{14}C]*myo*inositol in the supernatant of cell suspension in the incubation buffer. The rate calculated ($\times 10^{-4}$ mM · min^{-1} of cell water) has a within-day CV of 15.1% and a between-day CV of between 18 and 24%.

Calculation of the kinetics of *myo*inositol uptake. With no available specific transporter inhibitor, the dissociation of passive influx and transporter-mediated influx was estimated using Marquardt's method (14) for nonlinear fitting the Michaelis Menton formula:

$$V = \frac{V_{max}(S)}{K_m + (S)} + K(S)$$

where V is the rate of uptake of *myo*inositol in $\times 10^{-4}$ mM · min^{-1}, (S) is the concentration of *myo*inositol in the extracellular medium in μM, K is the rate constant of passive influx in $\times 10^{-3}$ min^{-1}, and V_{max} and K_m are the Michaelis Menten kinetic parameters of the saturable component of *myo*inositol influx. Calculations were made by one of us (L.L.N.) without knowledge of diabetic or neuropathic state.

Measurement of plasma and intracellular *myo*inositol concentration. Plasma *myo*inositol concentration was measured following its mixture with absolute alcohol and an internal standard of α-methyl mannoside. Samples were spun, the supernatant removed and evaporated under reduced pressure. The samples were transformed to their trimethyl silyl derivatives using a mixture of pyridine, hexamethyldisilazane, and trimethylchlorosilane, in the ratio 10:2:1, as described previously (15). Water was added to the samples, and the *myo*inositol was extracted into cyclohexane. The samples were mixed and the supernatant removed into glass vials. The samples were run on a 25-m cross-linked methyl silicone column on a Hewlett-Packard 5890 gas chromatography machine using helium as a carrier gas. The intra-assay CV for *myo*inositol concentration was 5.9%; the interassay CV for plasma *myo*inositol was 15.2%.

Leukocytes were also collected for intracellular *myo*inositol concentration assay in 14 diabetic and 7 nondiabetic subjects. After incubation in the water bath, an aliquot of cell suspension (460 μl) was washed twice in cold stop solution, and two 30-μl aliquots were taken from the homogenate for cell volume calculation. The other 400 μl was centrifuged, and the pellet dried and frozen. Samples were transported to the Imperial Chemical Industry Laboratory in dry ice and freeze dried, then handled in the same way as the plasma samples. The interassay CV for the intracellular *myo*inositol was 15.3%.

Comparison of cell volume by diabetic status. In separate experiments, 15 diabetic and 15 nondiabetic subjects were venepunctured, leukocytes were extracted and cell volume estimated, as described above. The cell pellet was subsequently heated and the dry weight measured on a Sartorius balance.

Statistics. Results are reported as means ± SD or median (ranges in parentheses). Linear regression by the method of least squares and paired *t* tests were performed. Other statistics (Mann-Whitney, median test) were calculated with SPSS. All *P* values were twotailed.

RESULTS

The cellular volume as measured isotopically in relation to the dry weight of cell was similar between diabetic and nondiabetic subjects (2.51 ± 0.58 vs. 2.46 ± 0.35 L · kg^{-1}, respectively). This was despite fasting hyperglycemia (12.5 ± 4.2 vs. 5.1 ± 0.4 mM) and a higher HbA_{1c} (9.5 ± 2.2 vs. 6.0 ± 0.6%).

Of the 41 diabetic subjects participating, 23 (56%) agreed to venepuncture and nerve conduction study. Of the others, 15 refused or were unable to take part and 2 had moved. Table 1 shows the characteristics of the participants and that diabetic and nondiabetic subjects were well matched for age and body mass index. Those attending for venepuncture were similar to those not attending. Those with diabetes had significantly higher systolic blood pressure and fasting glucose ($P < 0.001$).

Figure 1 shows that the *myo*inositol influx rates were higher in diabetic subjects at all concentrations of extracellular *myo*inositol besides 150 μM. The HbA_{1c} correlated positively with influx rates among diabetic subjects at 3 μM ($R_s = 0.599$, $P < 0.005$), 50 μM ($R_s = 0.429$, $P < 0.05$), 100 μM (0.427, $P < 0.05$) and 150 μM (0.485, $P < 0.05$) and the fasting glucose correlated negatively with the *myo*inositol influx rate at 3 μM ($R_s = -0.571$, $P < 0.05$) among nondiabetic subjects. The calculated K_m, V_{max}, and passive components were not significantly different between diabetic and nondiabetic subjects, and none correlated with either the glucose or HbA_{1c}. However, V_{max}:K_m ratio was significantly lower in nondiabetic subjects ($P < 0.01$) and correlated significantly with the HbA_{1c} in diabetic subjects ($R_s = 0.541$).

Of those with diabetes, six had no neurophysiological evidence of neuropathy, 7 had mild neuropathy, and 10 had moderate neuropathy (Table 2). Three of those thought clinically to have no neuropathy had abnormal nerve conduction tests. No significant differences were noted among the three neuropathy groups in HbA_{1c}, fasting glucose, years of diabetes, age, blood pressure, plasma-free insulin, or insulin units used although nonneuropaths had a higher body mass index than neuropaths (25.0 [22.6–27.9] vs. 23.3 [20.8–29.1], 22.5 [19.5–30.4] kg · m^{-2} respectively, $P < 0.05$). No significant difference in either rates of *myo*inositol influx or the

TABLE 1
Characteristics of patients in study

	Nondiabetic	Diabetic
n	13	23
Men (%)	46	63
Age (yr)	50 ± 6	50 ± 8
BMI (kg · m^{-2})	23.1 ± 2.5	23.8 ± 2.7
Height (cm)	170 ± 11	172 ± 10
Years of diabetes*		29 ± 6
Smokers (%)	62	52
Systolic blood pressure	109 ± 12	129 ± 19†
Diastolic blood pressure	71 ± 11	72 ± 9
Insulin dosage (U/kg)*		37 ± 9
Glucose (mM)	4.6 ± 0.6	11.0 ± 4.9†*
HbA_1 (%)*		9.9 ± 1.6
Insulin (pM)	40.2(30.6–96.0)	48.0(19.2–221.4)
$V_{max} \times 10^{-4}$ · mM · min^{-1}	7.9(4.3–22.7)	11.6(6.9–19.1)
K_m (μM)	60.2(23.1–196.5)	43.7(15.0–185.3)
B (measure of passive flux)	1.9(1.1–3.8)	2.2(1.1–3.8)
$\times 10^{-3}$/min V_{max}:K_m ratio	0.16(0.13–0.19)	0.25(0.17–0.32)‡

Values are means ± SD or median (ranges in parentheses).
*Not applicable or not available for nondiabetic subjects.
†$P < 0.005$, ‡$P < 0.01$.

derived kinetic parameters was found among the three neuropathy groups (Table 3).

The plasma *myo*inositol concentration was 0.028 (0.026–0.032) mM in nondiabetic subjects and 0.026 (0.024–0.035) mM in diabetic subjects. The intracellular *myo*inositol concentrations were 0.33 (0.23–0.58) mM in nondiabetic and 0.34 (0.21–0.51) mM in diabetic subjects. No difference in plasma or intracellular *myo*inositol concentration was observed between neuropathic and nonneuropathic subjects, nor any correlation with other measurements.

Although no significant difference in the proportion of smokers was found among the three groups (none 17%, feet only 57%, feet and hands 60%), diabetic subjects who smoked had significantly lower sural nerve and ulnar nerve sensory amplitudes (2.0 [0.0–7.0] vs. 6.5 [0.0–12.0] and 2.0 [0.0–4.4] vs. 5.0 [0.0–8.0] μ*V*, $P < 0.05$, respectively).

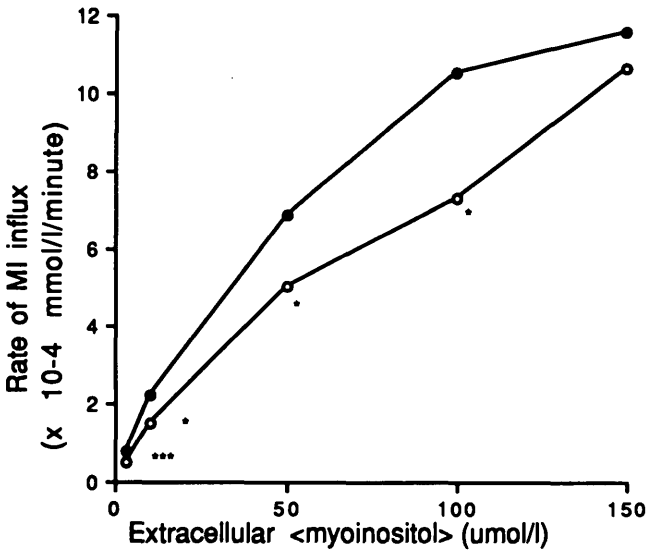

FIG. 1. Rate of [^{14}C]*myo*inositol uptake with increasing extracellular concentration of *myo*inositol in diabetic (*solid circles*) and nondiabetic subjects (*open circles*). ***$P < 0.005$, *$P < 0.05$.

DISCUSSION

As with human sural nerves (5) and erythrocytes (16), the intracellular *myo*inositol concentration in human leukocytes is similar in diabetic and nondiabetic subjects. This finding suggests that animal models with differences in intracellular *myo*inositol concentrations between diabetic and nondiabetic subjects may be inappropriate for the study of human *myo*inositol kinetics. The 10-fold difference in *myo*inositol concentration between plasma and the intracellular milieu supports the existence of a *myo*inositol transporter, unlike in the human erythrocyte (16) where the intracellular *myo*inositol concentration is low.

Our study shows that *myo*inositol influx mechanisms in cells from living diabetic subjects are abnormal. The similarity in the passive component suggests that abnormality in *myo*inositol flux involves only the transporter or its control mechanisms. This could be due to increased affinity of the transporter or increased number of transporters, because the diabetic K_m is not significantly lower and the V_{max} not significantly higher than in nondiabetic subjects. The raised V_{max}: K_m ratio suggests that there is an increased turnover rate of *myo*inositol at each transport site rather than an increase in the total number of transporter sites in diabetes. *myo*Inositol influx will be particularly affected as it is working near the K_m of the transporter.

We have previously shown that hyperglycemia inhibits *myo*inositol influx (8), and the increased uptake in washed diabetic leukocytes suggests the existence of a mechanism to compensate for the competition between plasma glucose and *myo*inositol, thereby resulting in increased flux once the glucose is removed. Such an adaptation would limit the deleterious effects of glucose on *myo*inositol uptake. The correlation of influx rates with

TABLE 2
Nerve conduction by neuropathy group

Neuropathy (*n*)	None (6)	Feet Only (7)	Feet and hands (10)
Sensory: sural			
Amp (μV)	9.0 (6.0–12.0)	4.1 (2.0–8.7)	0.0 (0.0–3.0)
Peak lat (ms)	4.6 (4.1–5.6)	4.7 (3.4–5.4)	0.0 (0.0–6.1)
Sensory: median			
Amp (μV)	4.3 (3.0–9.0)	4.2 (0.0–7.0)	3.0 (0.0–4.4)
Peak lat (ms)	3.5 (3.3–5.6)	4.1 (0.0–5.1)	4.3 (0.0–5.8)
Sensory: ulnar			
Amp (μV)	5.0 (4.4–8.0)	3.0 (0.0–5.0)	1.0 (0.0–5.0)
Peak lat (ms)	3.2 (3.1–4.2)	3.3 (0.0–3.9)	1.6 (0.0–4.2)
Motor: posterior tibial			
CMAP (mV)	15.2 (5.6–18.5)	7.6 (1.6–18.2)	5.3 (1.2–17.0)
DML (ms)	4.6 (3.3–6.5)	5.3 (3.8–6.4)	5.8 (4.1–9.5)
FWL (ms)	53.5 (45.3–57.0)	55.2 (46.1–68.0)	58.5 (0.0–70.5)
Motor: ulnar			
CMAP (mV)	10.4 (6.7–13.7)	11.4 (7.2–13.8)	11.7 (3.4–15.2)
DML (ms)	3.0 (2.3–3.5)	3.5 (3.0–3.8)	3.6 (2.7–5.1)
FWL (ms)	28.2 (26.0–32.6)	28.9 (28.5–35.0)	33.2 (28.3–37.9)
Wrist: thermal threshold testing (°C)			
Warming	0.18 (0.08–3.45)	0.53 (0.05–2.97)	0.28 (0.05–1.05)
Cooling	0.12 (0.08–0.40)	0.15 (0.13–0.50)	0.15 (0.08–0.80)
Foot: thermal threshold testing (°C)			
Warming	1.43 (0.18–3.05)	0.57 (0.50–2.35)	2.15 (0.75–5.40)
Cooling	0.39 (0.08–2.50)	0.22 (0.15–0.65)	0.99 (0.15–4.00)

Values are median (ranges in parentheses). All studies were performed on the subjects' right sides. Peak lat, peak latency (m), Amp, amplitude; DML, distal motor latency; FWL, F-wave latency; CMAP, compound muscle action potential.

HbA_{1c} in diabetic subjects rather than the glucose concentration at the time of venepuncture suggests that long-term glycemic control has a more permanent influence on the transporter kinetics than the ambient glycemia.

The failure to demonstrate differences in either the rates of transport or the derived kinetic parameters among diabetic subjects with various degrees of neuropathy suggests that differences in *myo*inositol uptake are not responsible for the development of diabetic neuropathy. This is supported by the similarity in plasma and intracellular *myo*inositol concentrations in diabetic and nondiabetic subjects and would be consistent with the failure to improve neuropathy after treatment with dietary supplementation of *myo*inositol (17). However, these findings do not exclude inherited or acquired sensitivities to the effects of abnormal *myo*inositol flux in diabetes. The lower sural nerve and ulnar nerve sensory amplitudes in the subjects who smoked is similar to that previously reported (18), and diabetic nerves have been shown to be relatively hypoxic (19).

The exclusion of subjects on treatments for conditions other than diabetes may have resulted in the recruitment of a survivor group. After 20 yr with diabetes, most diabetic patients exhibit electrophysiological evidence (20) and up to 70% show clinical evidence (18) of neuropathy. Only 74% of this study group had neuropathy, supporting the suggestion of a survivor cohort. Further evidence for this comes from the similarity in glycemic control and blood pressure between those with and without neuropathy, suggesting that those with diabetic tissue damage associated with hypertension and poorer glycemic control may have been excluded from the study. Another possible source of error could arise from the altered intracellular volume in cells from hyperglycemic rather than euglycemic subjects; this was ex-

TABLE 3
*myo*Inositol influx rates and kinetics

Concentration (μM)	Neuropathy		
	None	Feet Only	Feet and Hands
3	0.91 (0.42–1.09)	0.68 (0.44–1.13)	0.71 (0.41–1.47)
10	2.32 (1.26–3.26)	1.31 (1.04–3.16)	2.31 (1.30–4.08)
50	7.01 (3.73–9.93)	6.90 (4.40–11.0)	5.92 (3.94–11.0)
100	11.1 (6.85–15.8)	8.84 (4.96–22.2)	10.3 (6.36–16.7)
150	11.7 (7.02–17.0)	11.9 (10.2–18.7)	10.2 (6.88–19.1)
K_m (μM)	40.1 (31.1–57.3)	86.1 (15.0–185.3)	41.1 (20.5–58.8)
V_{max}	10.8 (5.2–19.6)	15.8 (2.8–26.7)	8.5 (6.5–20.1)
B (measure of passive flux)	2.2 (1.6–3.8)	2.1 (1.6–3.2)	2.4 (1.1–2.6)
V_{max}:K_m ratio	0.27 (0.16–0.33)	0.19 (0.13–0.29)	0.25 (0.20–0.36)

Values are median (ranges in parentheses). V_{max} = $\times 10^{-4}$ mM/min. B = $\times 10^{-3}$/min.

cluded by comparing dry weight of cells per unit calculated volume in diabetic and nondiabetic cells.

In conclusion, *myo*inositol transport is abnormal in diabetes and is related to long-term glycemic control. However, the development of diabetic neuropathy is not directly attributable to differing *myo*inositol kinetics but is probably due to a complex relationship between smoking, genetics, hyperglycemia, and vascular disease.

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