Diabetic neuropathy in db/db mice develops independently of changes in ATPase and aldose reductase

A biochemical and immunohistochemical study

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Summary. ATPase activity was investigated in sciatic and optic nerves of female mutant diabetic C57Bl/Ks (db/db) mice and age-matched control mice (db/m and m/m). Nerves from animals aged 50, 70, 125, 180 and 280 days were assayed in vitro for ATPase activity in the presence or absence of ouabain: the ouabain-sensitive fraction contained Na⁺,K⁺-ATPase. Enzymatic activity was compared within and between age-matched groups. No significant difference in Na⁺,K⁺-ATPase activity was detected between the diabetic and control mice, whether expressed as $\mu\text{mol P}_i/\text{h}^{-1}$ formed per gramme wet weight or per nerve (protein content). The activity decreased by about 25% in both the sciatic and optic nerves of the oldest animals. These results were strikingly similar in all groups, regardless of the type of nerve examined, confirming that the development of neuropathy in this

animal model is unrelated to the postulated derangement of Na⁺,K⁺-ATPase activity. Among possible explanations, a lack of polyol pathway activation was investigated by staining the sciatic nerves of animals from all groups with the peroxidase-antiperoxidase procedure using a polyclonal antiserum raised against the enzyme aldose reductase. Histological sections of all nerves were consistently negative, suggesting that these animals actually lack the enzyme involved in activating the self-perpetuating metabolic cycle leading to deranged nerve function. The db/db mouse appears to present particular biochemical changes which merit attention with a view to clarifying the pathogenesis of diabetic neuropathy.

Key words: Na⁺,K⁺-ATPase, aldose reductase, sciatic nerve, optic nerve, diabetic mutant mice, immunohistochemistry.

Early morpho-functional abnormalities in experimental diabetic neuropathy are commonly attributed to diminished Na⁺,K⁺-ATPase activity with intra-axonal Na⁺ accumulation and ensuing paranodal swelling. The pathogenic mechanisms leading to abnormal Na⁺,K⁺-ATPase activity may involve the enhancement in the flux of sorbitol and other metabolites of the polyol pathway across the axolemma as a consequence of the activation of the enzyme aldose reductase [1–6]; the inhibition of *myo*-inositol uptake; the alteration of phosphoinositide turnover and Na⁺,K⁺-ATPase activity, creating a potentially self-reinforcing cycle [1, 7, 8] which terminates with nerve fibre degeneration.

Such metabolic abnormalities, which are supposed to underlie the development and progression of human diabetic polyneuropathy as well, are currently exten-

sively investigated in a variety of animal models, as a source of new therapeutic approaches to this complication in man.

The diabetic mutant C57Bl/Ks (db/db) mouse, which develops a neuropathy similar to that accompanying human Type 2 (non-insulin-dependent) diabetes mellitus, has been widely studied. Affected animals present distal symmetrical neuropathy characterized by a reduction in nerve conduction velocity, mean axonal area, density of myelin and internodal particles, and alterations in the axonal transport of cytoskeletal proteins, noradrenaline and acetylcholinesterase [9–16].

However, recent studies have shown that peripheral nerves of diabetic mutant db/db mice may undergo neuropathic changes, susceptible to improvement by pharmacological treatment, in the absence of *myo*-inositol depletion

Fig. 1. Body weight gain of C57Bl/Ks female mice from 50 to 280 days of age. Diabetic (db/db) homozygotes are consistently heavier than age-matched control mice (m/m) throughout the life span. □ m/m mice; ▨ non-diabetic heterozygotes (db/m); ■ db/db mice

and Na^+, K^+ -ATPase deficit [5, 6, 17]. Since spontaneously diabetic mice seem to lack aldose reductase [5], the present study was designed (1) to investigate whether an early, transient impairment of Na^+, K^+ -ATPase activity could induce permanent neuropathy in C57Bl/Ks (db/db) mice; (2) to detect possible differences in nerve susceptibility, as represented by sciatic and optic nerves; and (3) to assess by immunohistochemistry either the absence of aldose reductase or its occasional localization and distribution.

Materials and methods

Animals

Age-matched control (m/m and db/m genotypes) and diabetic (db/db genotype) female mice of the C57Bl/Ks strain were obtained from Jackson Laboratories (Bar Harbor, Me, USA). Animals were killed by decapitation at 50, 70, 125, 180 and 280 days of age. To ascertain the diabetic and non-diabetic state, non-fasting blood glucose was measured by the hexokinase method (Boehringer, Mannheim, FRG); HbA_{1c} was also evaluated (Glycohemoglobin- HbA_{1c} kit, Sigma Chemical Co., St. Louis, Mo, USA).

Biochemistry

For the ATPase assay, optic nerves were dissected out starting from the posterior face of the eyebulbs to the anterior border of the optic tectum; sciatic nerves were surgically exposed at mid-thigh and dissected out from the sciatic notch to the popliteal fossa. Nerves were then weighed and homogenized in a chilled solution containing 0.25 mol/l sucrose, 1.25 mmol/l EGTA and 10 mmol/l Tris, pH 7.5 (at a 1:20 weight/volume (w/v) ratio for sciatic nerves and in 75 μl for optic nerves), in a glass-glass Potter-Elvehjem homogenizer (SAVI, Milano, Italy). Aliquots were frozen and stored at -80°C until assayed.

Total (composite), ouabain-sensitive (Na^+, K^+ -) and ouabain-insensitive (Mg^{++} -)ATPase activity were determined spectrophotometrically by the coupled-enzyme assay of Scharschmidt et al.

[18] using ouabain, adenosine-5-trisphosphatase (vanadate < 10 parts per million), phosphoenolpyruvate, NADH, pyruvate kinase and lactate dehydrogenase (Boehringer). Other reagents were pure grade (Carlo Erba, Milano, Italy). Mg^{++} -ATPase activity was expressed as specific activity ($\mu\text{mol P}_i \cdot \text{h}^{-1} \cdot \text{mg}^{-1}$ protein) released in the presence of ouabain (3 mmol/l final concentration). Na^+, K^+ -ATPase activity was calculated by subtracting the activity assayed with ouabain from that assayed without. Proteins were determined according to Lowry et al. [19] with bovine serum albumin as standard (Sigma Chemical Co).

Immunohistochemistry

The polyclonal antibody used in the present study was a kind gift from Dr. A.A.F. Sima (Winnipeg, Canada). The antiserum was raised against aldose reductase purified from testes of non-diabetic BB rats [22] and further identified by comparing aldose reductase molecular weight with that of standard protein markers on sodium dodecylsulfate polyacrylamide gels. The antibody was absorbed with liver powder before immunohistochemistry by the peroxidase-anti-peroxidase (PAP) procedure.

The investigations were carried out only on sciatic nerves dissected out from both normal and diabetic animals. Nerve specimens were fixed by immersion in Bouin's fixative, dehydrated and embedded in paraffin. After deparaffinization, endogenous peroxidase activity was blocked by soaking the sections in absolute methanol and 0.3% H_2O_2 for 30 min. Sections (5 μm -thick) were then incubated with normal goat serum for 2 h. The sections were washed in phosphate-buffered saline and immersed overnight at 4°C in the primary antibody (diluted 1:50), then at room temperature for 2 h in a solution (1:40) containing goat anti-rabbit IgG (Sternberger-Meyer Immunocytochemicals, Inc., Jarrettsville, Md, USA). Finally the sections were treated with rabbit PAP complex (Sternberger-Meyer, diluted 1:200) at room temperature for 1 h. The reaction product was developed by incubation in freshly prepared 0.05% diamine benzidine solution containing 0.01% H_2O_2 .

Statistical analysis

Statistical significance of any differences was evaluated by two-way analysis of variance; multiple comparisons were done by Tukey's highly significant difference test. All values are expressed as mean $\pm$ SEM.

Results

The diabetic mice (db/db) were obese and almost twice as heavy as the control animals (m/m and db/m) at all ages studied (Fig. 1); the weight difference reached statistical significance only in growing animals (50 days of age).

Blood glucose concentrations in the various groups of mice are given in Figure 2. Hyperglycaemia was already present in the diabetic mice by 50 days of age, reaching approximately 22.25 mmol/l at later ages. Control animals had blood glucose levels consistently around 6.40 mmol/l. The diabetic animals had elevated HbA_{1c} as early as 50 days of age. HbA_{1c} became almost twice that of the control animals at 280 days of age (Fig. 3). Protein content in the nerves (expressed per gramme wet tissue in the sciatic samples, or per unit volume in the optic nerve) rose from age 50 to 125 days, levelling off at later ages (data not shown).

Fig. 2. Blood glucose levels in C57Bl/Ks normal (m/m), non-diabetic heterozygotes (db/m) and diabetic homozygotes (db/db). In the first two groups blood glucose remains constant throughout the animal's life. □ m/m mice; ▨ db/m mice; ■ db/db mice

Fig. 3. HbA_{1c} levels in C57Bl/Ks mice aged 50 and 280 days. The increase at later age correlates with that of blood glucose levels. □ m/m mice; ▨ db/m mice; ■ db/db mice

ATPase activities, measured in the absence (composite) and presence of ouabain (to distinguish ouabain-resistant or Mg^{++} -ATPase from ouabain-sensitive or Na^+, K^+ -ATPase) in sciatic and optic nerves of 50, 70, 125, 180 and 280 day-old mice were basically similar in the age-matched groups. Na^+, K^+ -ATPase activity/mg protein from animals of all ages is shown in Figures 4 and 5. Throughout the study enzymatic activity maintained a plateau in both nerves until 125 days of age, then dropped slightly with a significant reduction at 280 days in all animals, whether normal homozygotes (m/m), heterozygotes (db/m) or diabetic homozygotes (db/db). Compared to diabetic homozygotes, in m/m and db/m mice values in the sciatic nerves were slightly lower than those of the optic nerves, but no appreciable differences were detected for either nerve between individual groups of corresponding ages.

Fig. 4. Na^+, K^+ -ATPase activity in the sciatic nerves of C57Bl/Ks normal (m/m), non-diabetic (db/m), diabetic (db/db) mice. The enzymatic activity of diabetic samples is slightly higher at all ages studied. □ m/m mice; ▨ db/m mice; ■ db/db mice

Fig. 5. Na^+, K^+ -ATPase activity in optic nerves of C57Bl/Ks normal (m/m), non-diabetic (db/m), diabetic (db/db) mice. A slowly progressing decline in activity is seen at later ages in all three genetic groups. □ m/m mice; ▨ db/m mice; ■ db/db mice

Activities expressed as gramme/nerve/wet weight gave similar results; however, on account of the difference in protein content in younger, still-growing animals, the relative values of ATPase activity varied within the groups. For instance, in the optic nerves of 50-day-old db/db mice activities were 28.4 ± 2.1 , 9.81 ± 0.7 and $19.4 \pm 1.7 \mu\text{mol Pi} \cdot \text{h}^{-1} \cdot \text{mg}^{-1}$ for composite, Na^+, K^+ - and Mg^{++} -ATPase respectively, and 36.4 ± 3.9 , 9.28 ± 0.9 and $25.0 \pm 3.3 \mu\text{mol Pi} \cdot \text{h}^{-1} \cdot \text{mg}^{-1}$ in 125-day-old mice. Such differences reflect about a 1.3-fold increase (from 1.97 to 2.70 mg/ml) in protein content over a period of 2.5 months.

Composite and Mg^{++} -ATPase activities followed the same pattern as Na^+, K^+ -ATPase over this period. The ranges of activity were 4.2–7.5 U and 2.6–4.9 U for the sciatic nerve, and 8.2–14.5 and 2.6–4.6 for the

Fig. 6a and b. Aldose reductase immunohistochemistry in the sciatic nerve of a 280-day-old diabetic mutant (db/db) mouse. **a** preimmune serum. **b** anti-aldose reductase serum. Neither Schwann cell perinuclear regions (S) nor nodes of Ranvier (arrows) show particular immunostaining over the background. $\times 1000$

optic nerve, respectively. Na^+, K^+ -ATPase activity turned out to be consistently higher (3–5 fold) in the optic nerve than in the sciatic (Fig. 5). This increase reflected specifically those of Na^+, K^+ -ATPase since Mg^{++} -ATPase levels remained similar (see above) in both nerve types.

Staining reactivity on cross- and longitudinal sections processed for PAP immunohistochemistry showed that, in contrast to non-diabetic BB rats [20], galactose-fed rats [21], and alloxan- and streptozotocin-diabetic rats [22], sciatic nerves of diabetic mutant (db/db) mice and those of the normal homozygotes (m/m) and heterozygotes (db/m) were consistently negative (Figs. 6a, b). These results confirmed that they actually lack aldose reductase, at least in the form recognizable by the polyclonal antibody used in this study.

Discussion

The present study addressed the hypothesis that, as generally accepted for the experimentally diabetic rat, a reduction in ATPase activity may be involved in the development of neuropathy in the C57Bl/Ks (db/db) mice, a well-characterized genetic rodent model for non-insulin-dependent diabetes mellitus [23].

The activity of ATPase was measured in female diabetic (db/db) and control (m/m and db/m) C57Bl/Ks mice in the 50–280 day age range. Special attention was paid to whether nerves with different characteristics, such as the optic and the sciatic, undergo similar changes throughout the period under study, regardless of the diabetic status.

The findings indicate that there are only minor differences in this enzymatic activity in relation to age and diabetic status; no deficit was present in mice with spontaneous diabetes at any age in either optic or sciatic nerves, though a 20–30% decline was measured in the oldest animals.

The assumption that Na^+, K^+ -ATPase plays a crucial role in the pathogenesis of diabetic neuropathy stems from the abnormalities in the turnover of phosphoinositides which lead to reduced ATPase activity; this may activate a self-perpetuating cycle which results in progression of axonal damage [1]. It has been demonstrated that diabetes in C57Bl/Ks mice gives rise to various functional and morphological changes affecting peripheral nerves, such as reduction in nerve conduction velocity and mean axonal area, alteration in axonal transport of cytoskeletal proteins, noradrenaline and acetylcholinesterase, and decrease in density of myelin and internodal membrane particles [9–16]. However, these changes are amenable to hormonal and pharmacological treatment [9, 10, 13].

The development of neuropathy in this animal model seems unrelated to the other biochemical changes reported in rats made diabetic with either alloxan or streptozotocin or in the spontaneously diabetic BB rat [5, 6]. The peripheral nerves of db/db mice appear to lack the enzyme aldose reductase since, unlike other animal models, they do not accumulate fructose and sorbitol, the metabolites of the polyol pathway. The present results show that this lack is genuine, since sciatic nerves of either normal or diabetic mice of the C57Bl/Ks strain do not immunostain with an anti-aldose reductase polyclonal antiserum.

Recent works have questioned whether a massive accumulation of polyols is necessary for the progression to secondary complications in diabetes. For instance, Tomlinson et al. [6, 24] showed that overstimulation of the polyol pathway by feeding db/db mice with galactose led to accumulation of dulcitol, suggesting the presence of a certain “reductase” sensitive to treatment with an aldose reductase inhibitor. However, other studies have failed to reveal consistently low *myo*-inositol levels. In addition, the pattern of alteration of polyphosphoinositides in db/db mice seems different from that in other animal models, in spite of similar features in peripheral myelin protein phosphorylation [7, 8, 25, 26]. As an example, in sciatic nerve of streptozotocin-diabetic rats, a known model for Type 1 (insulin-dependent) diabetes mellitus, and in the same nerve of the Wistar fatty rat, a prospective model for Type 2 diabetes, there is a stimulation of [^{32}P] incorporation into polyphosphoinositides that is not seen in db/db mice; in fact, in these latter animals the uptake into phosphatidic acid is elevated [7, 25, 26].

A recent study by this laboratory on phospholipid phosphorylation in streptozotocin-diabetic rats showed that gangliosides were able to normalize the altered turnover of phosphoinositides, including phosphatidic acid [27]. In view of the fact that, in C57Bl/Ks (db/db) mice, ganglioside treatment improves electrophysiological and morphometric par-

ameters and axonal flow [15, 16], but only once peripheral neuropathy is present [12, 13], it would be interesting to analyse the effect of this drug on phosphoinositide turnover as well.

Taken together, these findings support the use of each different animal model as a means of identifying and clarifying the various pathogenic factors contributing to the progression and development of human diabetic polyneuropathy. Since this complication may develop in C57Bl/Ks mice independently of any significant change in aldose reductase and Na^+, K^+ -ATPase activity, this mouse strain is a particularly good model for investigating alternative mechanisms leading to nerve pathology in diabetes.

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