Amino acid uptake by dorsal root ganglia from streptozotocin-diabetic rats

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SUMMARY Dorsal root ganglia from streptozotocin-diabetic rats and age- and weight-matched control animals were incubated with 4-amino-3H-butyric acid. A significant reduction in uptake was observed in the diabetic animals. The incorporation of 3H-leucine into protein was also significantly reduced but the results did not allow a conclusion as to whether this was a secondary or an independent effect. These findings are discussed in relation both to the abnormalities known to develop in diabetic rats and to the causation of human diabetic neuropathy.

Since the first demonstration of reduced nerve conduction velocity in alloxan-diabetic rats by Eliasson,1 numerous studies have been undertaken on alloxan or streptozotocin-induced diabetes in rats and rabbits, and more recently on spontaneous diabetes in animals, in an attempt to elucidate the cause of diabetic neuropathy in man.

A number of metabolic defects have been demonstrated in peripheral nerve in chemically-diabetic rats. Sorbitol is known to accumulate,2 related to augmentation of the sorbitol pathway consequent upon the hyperglycaemia. The concentration of myo-inositol is also known to be reduced.3 These observations have been separately implicated as having a possible causal relationship to the reduced nerve conduction velocity. Prevention of sorbitol accumulation with an aldose reductase inhibitor will improve conduction velocity.4,5 without change in the level of hyperglycaemia. Dietary supplementation with myo-inositol to restore nerve myo-inositol levels will also improve nerve conduction velocity.6 Although this is difficult to demonstrate in mature animals that have ceased growing,6 Additionally, it is known that Na+/K+ ATPase activity is reduced in diabetic nerve.7 Recent observations suggest that it may be possible to link these findings. Sorbitol accumulation appears to be a factor responsible for the reduction in nerve myo-inositol concentration,8 in addition to competitive inhibition by glucose of Na-dependent myo-inositol uptake.9 The changes in phospholipid-dependent membrane-associated ATPase activity provide a potential means of relating defective inositol phospholipid metabolism, reduced nerve conduction velocity, and impaired energy utilisation.10-12 It has been established that myo-inositol administration will prevent the reduction in Na+/K+ ATPase activity and that the reduced energy utilisation in endoneurial preparations of streptozotocin-diabetic rat nerve is restricted to the fraction that can be inhibited by ouabain and which is therefore attributable to Na+/K+ ATPase activity. Lastly, it has recently been found that the impaired Na+/K+ ATPase further interferes with Na-dependent myo-inositol uptake, thus producing a self-reinforcing cycle of compromised myo-inositol and Na+/K+ ATPase function in diabetic peripheral nerve.13

Another approach to the causation of diabetic neuropathy stems from the observation that nerve fibre diameter fails to show the normal increase during growth in streptozotocin-diabetic rats4 and genetically diabetic mice.14 This growth defect is prevented by insulin treatment.15 It has been shown that the slow component a of axonal transport is impaired in streptozotocin-diabetic rats.17 The hypothesis was therefore advanced that defective perikaryal synthesis of protein18 or reduced axonal transport19 may lead to the development of axonopathy. The present investigation was accordingly undertaken to examine amino acid uptake and incorporation into protein by diabetic dorsal root ganglia.
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Methods

Diabetes was induced in mature male Wistar rats weighing 450–700 g by the intraperitoneal injection of a buffered solution of streptozotocin at a dosage of 65 mg/kg. Diabetic animals and controls, paired by age and weight at the time of induction of diabetes, were maintained in plastic metabolic cages on 41B Oxoid diet (Lillico) with water ad libitum.

At biopsy under ether inhalation anaesthesia, laminectomy was performed and both first sacral dorsal root ganglia and the caudal two pairs of lumbar ganglia were removed under an operating microscope and transferred to the incubation medium. At each session, the ganglia from one diabetic and one control animal were processed. After excision, the three pairs of ganglia were placed in a petri dish containing 5 ml of Hank's solution (pH 7.2–7.4). Any extraneous pieces of spinal root, blood vessels, etc., were removed. The ganglia were transferred to a glass vial containing 5 ml of medium 199 (BDH Chemicals) with NaHCO₃, and oxygenated by blowing through a mixture of 95% O₂ and 5% CO₂ for 30 s.

For the observations on leucine uptake, 5 μCi of L-[4, 5-³H] leucine (Amersham International) were then added to the sample tube, which was incubated in a shaking water bath at 37°C for 3 h. The subsequent method for extraction of the proteins follows that of Cavanagh and Chen with some modifications. Incubation was stopped by running in 5 ml of 10% (w/v) trichloroacetic acid (TCA) and the tube centrifuged at 15,000 g for 15 min. All subsequent centrifugations were the same. The supernatant was discarded and 5 ml of cold (4°C) 10% TCA added. The ganglia were then macerated in a homogeniser and the tube centrifuged. The supernatant was discarded and the deposit suspended in 5 ml of cold TCA and again centrifuged. This was repeated twice. The third centrifugation, the deposit was washed in 5 ml of an ethanol/ether mixture (3:1 v/v) and centrifuged, the procedure being repeated consecutively with 5 ml chloroform/methanol (2:1 v/v) and then 5 ml ether. The deposit was resuspended in 5 ml of 5% TCA, heated in an oven at 90°C for 15 min and centrifuged, this procedure being repeated once. The deposit was finally washed in 5 ml of acetone and, after further centrifugation, resuspended in ether and allowed to dry. The residue was carefully scraped into a counting vial, using an ether wash if necessary, weighed, and 0.2 ml of distilled water added followed by 1 ml of NCS solubiliser. The vial was incubated in a water bath at 45°C until the tissue had dissolved and, after cooling, 4 ml of scintillation fluid (POPOP/POPOP mixture) added. Counting was undertaken in a Packard β counter after the tube had been allowed to stand in the dark for about 1½ h for the initial fluorescence to subside. Observations on the ganglia from four control animals showed that the uptake of ³H-leucine was linear after the 3 h incubation period (fig).

For the observations on the uptake of 4-amino butyric acid, 5 μCi of 4-amino-⁴-[2,3,5-³H] butyric acid (Amersham International) were added to the sample tubes containing the diabetic and control dorsal root ganglia, which were then incubated for 3 h at 37°C. Incubation was stopped by adding 5 ml of 10% trichloroacetic acid and, after centrifugation, the ganglia were washed 3 times in isotonic saline with centrifugation at 1500 g for 15 min and then dried of surface fluid. After weighing, they were solubilised as already described and counted.

The results both for 4-amino butyric acid uptake and ³H-leucine incorporation were compared between the diabetic and control animals using the Wilcoxon rank sum test for paired samples.

Results

Leucine uptake

Observations were made on 10 diabetic and 10 control animals. The duration of the diabetes was 2–4 months. Plasma glucose a the time of sacrifice was 37.4 ± 1.3 mmol/l (mean ± SE; range 30.4–42.4) in the diabetic animals and 9.3 ± 0.2 mmol/l (range 8.7–20.6) in the controls.

The results for ³H-leucine uptake, expressed as counts/min/mg protein, are given in table 1. The counts for the diabetic and control ganglia were 2949 ± 524 (mean ± SE) and 4199 ± 710 respectively. The counts were consistently lower in the diabetic ganglia and are significantly different at the 1% level (Wilcoxon rank sum test for paired samples).

A control experiment was performed to estab-

![Graph](image_url)
lished that the $^3$H-leucine uptake represented incorporation into protein. Three pairs of ganglia from two normal rats were studied. Cycloheximide (1 mg/ml) was added to the incubation medium for the ganglia from one animal. For these ganglia the count obtained was 208/min/mg protein and for the untreated ganglia 6895/min/mg.

4-amino butyric acid uptake

Observations were made on 10 diabetic and 10 control animals. The duration of diabetes was again 2–4 months. Plasma glucose at the time of sacrifice was 36.8 ± 1.84 mmol/l (mean ± SE; range 28.2–42.2) in the diabetic animals and 8.7 ± 0.45 mmol/l (range 61–10-9) in the controls. The results for the uptake of 4-amino $^3$H-butyric acid (see table 2) expressed as counts/min/mg wet weight of tissue were 678 ± 145 (mean ± SE) for the diabetic animals and 1112 ± 256 for the controls. The counts were consistently less for the diabetic ganglia. When compared statistically, this difference is significant at the 1% level (Wilcoxon rank sum test for paired samples).

Discussion

Observations on streptozotocin or alloxan-induced diabetics in animals show an increased rate of protein degradation in most tissues. In the "chronic" state (that is diabetes of two months or more duration), a steady state is probably reached, if the animals are not losing weight, between synthesis and degradation.21 The present study has addressed the question of protein synthesis in the dorsal root ganglia of streptozotocin-diabetic rats. In other tissues, uptake of leucine or other radiolabelled amino acids has usually been found either to be reduced or within normal limits in diabetic animals. Bond21 noted a general decrease in incorporation in streptozotocin-diabetic mice for liver, kidney, heart and skeletal muscle. For neural tissues, Spritz et al22 23 found a reduced incorporation of $^14$C-leucine into the protein components of myelin from in vitro studies. Chihara24 reported that the incorporation of leucine into the retina of diabetic rabbits was reduced, as was the amount of radiolabelled protein transported along the optic nerve. Transport velocity was normal, suggesting that the reduced transport along the nerve was secondary to impaired protein synthesis in the ganglion cells. Sidenis and Jakobsen,25 during a study on axoplasmic transport, reported a 40% decrease in the incorporation of leucine when injected into the fifth lumbar ganglion of streptozotocin-diabetic rats, and the half-life of the labelled proteins in the ganglion did not differ from that of controls. Of particular interest in the present context is the observation of Burnham et al26 that insulin enhances the uptake of $^3$H-leucine and $^3$H-uridine by intact and dissociated fetal dorsal root ganglia.

Our present results have shown that the uptake of $^3$H-leucine into protein is diminished in the dorsal root ganglia of streptozotocin-diabetic rats. The inhibition by cycloheximide established that the $^3$H-leucine was incorporated into protein and that the results did not merely represent passive diffusion into the ganglia. Cycloheximide, at the concentration employed, has a marked inhibitory effect on protein synthesis in vitro.27 The perikaryal volume of dorsal root ganglion cells has been found to be less in streptozotocin-diabetic rats as compared with controls.28 It is probable that, as for fibre size, this represents a failure of the normal increase during development and is thus unlikely to be a complicating factor in the present experiments in view of the maturity of the animals. This study has shown that the uptake of the non-metabolisable amino acid, 4-amino butyric acid, is depressed in the diabetic ganglia. Further studies will be required to establish whether the reduced incorporation of leucine into protein is secondary to the impaired amino acid uptake or whether it is an independent effect. It is of interest that observations on rat liver parenchyma cells have demonstrated that the uptake of 4-amino butyric acid is energy dependent and that the addition of insulin to the culture results in an increased influx of the amino acid.

Axonal structural proteins are synthesised in the neuronal perikaryon and transported down the axon in slow component a. This component carries tubulin and the neurofilament polypeptide triplet,29 and its velocity has been shown to be reduced in streptozotocin-diabetic rats.18 Although Takenaka et al20 failed to detect any alteration in transport rate in L5 sensory fibres of diabetic rats, their results suggested that a reduced amount of neurofilament

Table 2 Uptake of 4-amino $^3$H-butyric acid by dorsal root ganglia from diabetic and control rats (counts/min/mg wet weight of tissue)

<table>
<thead>
<tr>
<th></th>
<th>Diabetic</th>
<th>Control</th>
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<tr>
<td>264</td>
<td>395</td>
<td>-131</td>
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<td>132</td>
<td>348</td>
<td>-216</td>
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</tr>
<tr>
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<td>3009</td>
<td>-1874</td>
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</tr>
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<td>1125</td>
<td>1416</td>
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</tr>
<tr>
<td>1206</td>
<td>1298</td>
<td>-92</td>
<td></td>
</tr>
<tr>
<td>Means:</td>
<td>678 ± 145 (SE)</td>
<td>1112 ± 256 (SE)</td>
<td>-434</td>
</tr>
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</table>
material was transported, implying reduced incorporation of the \(^{35}\text{S}\)-methionine which was injected into the ganglion. Sidenius and Jakobsen\(^1\) also found a reduction in the amount of label transported and showed that the reduction in transport velocity that they had detected could be corrected by insulin.

There is therefore good evidence for reduced perikaryal synthesis in neurons contributing fibres to the peripheral nerves, and a reduced delivery of structural proteins to the periphery of the axon. This is likely to explain the failure of peripheral nerve axons to increase in size normally in rats made diabetic before cessation of the growth period. If severe enough, it might lead to a distal axonal degeneration. It is so far uncertain whether the synthesis of cytosol proteins is also affected, but again a reduced delivery of enzymes to the periphery could lead to a distal axonopathy.

There are interesting parallels between the effects of streptozotocin-induced diabetes and severe protein deprivation in rats. Studies in our laboratory, as already stated, have demonstrated that one of the effects of streptozotocin diabetes is a maturational retardation of nerve fibre diameter.\(^4\) Rats continue to show an increase in nerve fibre diameter until about 9 months of age and this is impaired in diabetic animals. This effect has also been noted by Sugimura et al.\(^3\) It is of considerable interest that if rats are made diabetic with streptozotocin at the time of weaning, skeletal growth and increase in body weight are immediately retarded, whereas the increase in nerve fibre diameter is at first maintained. It later shows a deficit in comparison with controls. This parallels the effects produced by protein deprivation\(^5\) which, if severe, is known to result in distal axonal degeneration.\(^6\)

Recent observations by Mayer and Tomlinson\(^5\) have demonstrated that defective orthograde transport of choline acetyltransferase in the sciatic nerve of streptozotocin-diabetic rats can be prevented and reversed by treatment with an aldose reductase inhibitor. It was suggested that this may be mediated by correction of the myo-inositol deficit in peripheral nerve discussed in the Introduction. It was further proposed that reduced axonal transport secondary to the myo-inositol deficit could result in distal axonal degeneration. We are currently investigating the explanation for the reduced amino acid uptake by dorsal root ganglion cells. It will be important to establish whether similar mechanisms are involved.

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### References