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EFFECT OF EXPERIMENTAL DIABETES AND INSULIN ON PHOSPHATIDYL-INOSITOL SYNTHESIS IN RAT SCIATIC NERVE

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SUMMARY: The rate of incorporation of [2-^{3}H]myo-inositol into phosphatidylinositol was found to be significantly decreased in sciatic nerve from both alloxan and streptozotocin-diabetic rats. The rates of incorporation into phospholipid of tritiated serine and ethanolamine were unchanged while choline showed an upward trend in sciatic nerve from alloxan-diabetic rats. Insulin added *in vitro* significantly increased [2-^{3}H]myo-inositol incorporation into phospholipids by normal rat sciatic nerve; only small changes were recorded with high concentrations of glucose, and galactose. The results are discussed in relation to the physiological functions of phosphatidylinositol and the role of free myo-inositol in the regulation of cellular processes.

The peripheral nervous system undergoes marked structural and functional alterations in diabetes as exemplified by the segmental demyelination and Wallerian degeneration, and by the depressed motor nerve conduction velocity [1-3]. Certain parallelisms exist between the abnormalities arising in diabetes and galactosemia, with regard to the decreased motor nerve conduction velocity, the accumulation of products of the polyol pathway, sorbitol and fructose in diabetes and galactitol in galactosemia, and in the reported depression in the nerve content of myo-inositol [4-6]. Restoration of the normal motor nerve conduction velocity in diabetic animals is achieved with insulin treatment and also by supplementation of the diet with myo-inositol [7,8].

The metabolic changes occurring in peripheral nerve in diabetes include a depressed oxidation of glucose and a lowered rate of incorporation of glucose and acetate into lipid [9-11]. The lipid composition of nerve also alters in diabetes [12], and Eliasson [13] has reported a decrease in the *in vitro* rate of

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incorporation of radioactive precursors into some of the myelin lipids, cerebroside synthesis showing a particularly marked depression.

Phosphatidylinositol occupies a focal position linking certain of the multiple changes occurring in peripheral nerve in diabetes. Not only is this compound an important component of cell membranes and myelin, but it has also been implicated in ion movement in nervous tissue [14]. These observations, taken in conjunction with the evidence for a decrease in diabetes of myo-inositol, prompted the present investigation into the effect of diabetes on aspects of phospholipid synthesis, in particular of phosphatidylinositol, in rat sciatic nerve. The effect of hexoses and hexitols, known to accumulate in peripheral nerve in diabetes and galactosemia, on myo-inositol incorporation into phospholipid was also investigated.

METHODS

Animals

Alloxan diabetes: Adult male albino rats of the Wistar strain 200-250 g body weight, were fasted for 24 hours and then injected subcutaneously with alloxan-monohydrate 200 mg/kg body weight; thereafter insulin was administered (2 units of protamine zinc insulin daily for one week) and standard laboratory cube diet and water were allowed ad lib. The rats were used 2-3 weeks after cessation of insulin treatment.

Streptozotocin diabetes: Rats of similar age and weight were used. Diabetes was induced by intravenous injection of streptozotocin, 75 mg/kg body weight. No preliminary period of fasting or subsequent treatment with insulin was given, standard laboratory cube diet and water were allowed ad lib. The rats were used 2-3 weeks after induction of diabetes. In each case the control group comprised rats of the same age from the same colony.

Precursor incorporation studies

Excised sciatic nerve segments: The rats were killed by cervical dislocation, the sciatic nerves were removed, cleaned and weighed; closely similar standard lengths of nerve, approximately 25 mm, were used for each incubation. Each incubation flask contained 2 μCi of one of the following precursors with specific activities as shown in parentheses: myo-inositol (5 Ci/mmole), serine (19 Ci/mmole), ethanolamine (3.8 Ci/mmole) and choline (8.4 Ci/mmole). The incubation medium was Krebs-Ringer bicarbonate, the final volume was 2 ml, the gas phase O_2:CO_2, 95:5 and the time of incubation was 2 hours.

Paired sciatic nerves from normal rats were used in the study of the in vitro effects of insulin, sugars and sugar alcohols on the incorporation of [2-^{3}H]inositol into phospholipid, the concentrations used were: insulin, 0.25 units/ml medium; glucose, 10 mM and 30 mM; sorbitol, 5 mM; galactose, 20 mM and galactitol, 30 mM. At the end of the incubation period the nerves were removed from the medium and placed immediately in ice-cold 0.9% sodium

chloride. The total lipids were extracted by the method of Bligh & Dyer [15], the extracted lipid was dried and resuspended in toluene containing 5 g 2,5-diphenyloxazole/litre, and counted using a Packard Tricarb Scintillation Counter.

In one series of experiments in which the incorporation of [2-^{3}H]myo-inositol into phospholipids of normal and streptozotocin-diabetic rats was investigated, the extraction of phospholipids was by the Folch procedure followed by separation of the phospholipids by thin layer chromatography. As shown in Table 1, essentially similar results were obtained by the two extraction procedures. (We are grateful to Miss Sheila Morgan for her help with these experiments).

Broken cell preparation: The measurement of the incorporation of [2-^{3}H]myo-inositol into phospholipid in broken cell preparation from sciatic nerve was essentially as described by Prottey & Hawthorne [16] for pancreas. Sciatic nerve was homogenized in 9 volumes of medium containing 0.25 M sucrose, 20 mM triethanolamine buffer pH 7.4, 0.1 mM dithiothreitol and 0.5% bovine serum albumin (fatty acid free) using an all glass conical homogenizer. After dialysis for 1 hour, in the cold, against the same medium as that given above with the bovine serum albumin omitted, the homogenate was fractionated by centrifugation at 106,000 g for 45 min. The total pellet fraction was resuspended in the same initial volume of homogenizing medium containing bovine serum albumin and 0.1-0.3 ml of this preparation was used in a final incubation volume of 1.0 ml. The incubation medium contained: 16.6 mM potassium phosphate buffer pH 7.4, 13.3 mM potassium fluoride, 8 mM ATP/Mg^{2+}, 1 mM CTP, 1.66 mM glycerophosphate, 80 μM CoA, 1 mM myo-inositol containing 1 μCi [2-^{3}H]myo-inositol/ml medium. The incubation period was 1 hour at 37°, at the end of which lipid was extracted by the method of Bligh & Dyer [15] and counted as given above.

Materials

Radiochemicals were obtained from the Radiochemical Centre, Amersham, Bucks, England. The co-enzymes and substrates were from Boehringer Corporation Ltd., Lewes, Sussex. Sugar and sugar alcohols were from British Drug Houses, Poole, Dorset.

RESULTS

The incorporation of [2-^{3}H]myo-inositol into phospholipid of sciatic nerve was significantly depressed in both the alloxan-diabetic and streptozotocin-diabetic groups of rats (Table 1). In contrast, the incorporation of tritiated serine and ethanolamine into the phospholipid fraction was unchanged, while choline incorporation was increased by 37% in sciatic nerve from alloxan-diabetic rats. The addition of insulin to the incubation medium increased the rate of [2-^{3}H]myo-inositol incorporation into lipid in segments of sciatic nerve from normal rats by approximately 50%.

The rate of incorporation of [2-^{3}H]myo-inositol into phosphatidylinositol using particulate fractions from sciatic nerve homogenates in the presence of high concentrations of myo-inositol

Table 1. The effect of diabetes, insulin, hexoses and hexitols on phospholipid synthesis from various labelled precursors by rat sciatic nerve.

Precursor	Control	Diabetic	N	P
	pmole/g/h			
Effect of alloxan diabetes				
[2-^{3}H]myo-inositol	8.67±1.05	5.07±0.49	(6)	< 0.01
[3-^{3}H]serine	0.047±0.005	0.039±0.004	(6)	NS
[1-^{3}H]ethanolamine	0.345±0.061	0.390±0.045	(6)	NS
[Methyl-^{3}H]choline	0.182±0.029	0.250±0.018	(6)	0.06
Effect of streptozotocin diabetes				
[2-^{3}H]myo-inositol	8.69±0.95	5.99±0.47	(6)	< 0.01
Effect of insulin				
[2-^{3}H]myo-inositol	7.99±0.79		(4)	
+ insulin	12.1 ±1.2		(4)	< 0.05
Effect of hexoses and hexitols				
[2-^{3}H]myo-inositol	8.64±0.93		(12)	
+ 10 mM glucose	8.54±1.07		(12)	NS
+ 30 mM glucose	7.39±1.46		(4)	0.08
+ 20 mM galactose	7.57±0.90		(11)	< 0.05
+ 5 mM sorbitol	9.03±1.25		(4)	NS
+ 30 mM galactitol	8.45±1.68		(4)	NS

The values represent the rate of incorporation of each tritiated precursor into a total phospholipid fraction. Each incubation contained 2 µCi of precursor with the following specific activities: myo-inositol, 5 Ci/mmole; serine, 19 Ci/mmole; ethanolamine, 3.8 Ci/mmole and choline, 8.4 Ci/mmole. The effect of insulin, 0.25 units/ml medium; glucose, 10 mM and 30 mM; sorbitol, 5 mM; galactose, 20 mM and galactitol, 30 mM, added *in vitro* to the incubation medium, on the rate of incorporation of [2-^{3}H]myo-inositol into total phospholipid is also shown. Each value is the mean ±SEM, the number of separate experiments are shown in parentheses. Fisher's P value is calculated from t independent in the case of alloxan and streptozotocin diabetes, and t dependent in the *in vitro* studies on the effects of insulin, hexoses and hexitols, where paired sciatic nerves from the same animal were used.

and cofactors was considerably greater than that found in excised nerve segments. Further, no significant differences were found between the control and diabetic rat peripheral nerve with regard to the overall rate of incorporation by the particulate fractions, which were, 15 ± 2 and 17 ± 2 nmoles/g/hr (mean $\pm$SEM of 4 pairs).

The incorporation of [2-^{3}H]myo-inositol into phospholipid was not significantly altered by high concentrations of sorbitol or galactitol added to the medium, however, high concentrations of galactose (20 mM) and glucose (30 mM) produced a small decrease of 12% and 14% respectively in the rate of incorporation of labelled myo-inositol into phospholipid.

DISCUSSION

The present results show that there is a decrease in the rate of incorporation of myo-inositol into phosphatidylinositol in sciatic nerve segments from diabetic rats at a stage where it is known that there is a decline in both the nerve content of free myo-inositol and in the motor nerve conduction velocity [6-8]. The depression in the rate of [2-^{3}H]myo-inositol incorporation into phospholipids in the present experiments could be due to one or more of a number of factors, including alterations in the transport of myo-inositol and changes in the activities of enzymes involved in phosphatidylinositol synthesis.

The balance of evidence would seem to favour the former hypothesis. It is known that most tissues, including peripheral nerve, contain concentrations of free myo-inositol that are considerably higher than those of plasma and the active concentration of this compound has been shown to occur in nervous tissue, lens and kidney [17-21]. The present results, showing that in diabetic rats there is a fall in the myo-inositol incorporation into phosphatidylinositol in the intact nerve segments but an unchanged rate in broken cell preparations, is in accord with the hypothesis that a depression in myo-inositol transport occurs in diabetes. Similarly, the elevated rate of myo-inositol incorporation into phospholipid when insulin is added in vitro to segments of normal sciatic nerve is also compatible with regulation of transport of this compound by insulin. Similar findings have been reported for isolated adipocytes [22] again pointing to membrane phospholipids as an important locus of insulin action. In this context it may also be noted that Field & Adams [23] showed that insulin enhanced

glucose uptake in isolated sciatic nerve and that the facilitated penetration of selected non-utilisable pentoses conformed to the pattern of stereospecificity characteristic of insulin action on adipose tissue and muscle. Sciatic nerve would thus appear to have many of the properties of insulin-sensitive tissues with regard to rate-limitation imposed by transport processes and regulation of these by insulin.

Although somewhat controversial [24], the observations of Greene et al. [8] that the addition of 1% myo-inositol to the diet of diabetic rats raised both the nerve content of myo-inositol and improved nerve function, as shown by the motor nerve conduction velocity, are indicative of a depression in intracellular myo-inositol being directly related to nerve function. Recent electron microscopy studies have shown interesting structural changes in nerve membrane components in diabetes and their reversal by insulin and myo-inositol administration [25].

Comparison of the present results with those of Spritz et al. [26] show that different profiles of change among the component phospholipids are found depending upon the precursor used and the cell component isolated. In the present studies only labelled myo-inositol incorporation decreased in diabetes, while Spritz et al. [26] reported decreases in the labelling of myelin, phosphatidylcholine, phosphatidylethanolamine and phosphatidylserine in young diabetic rats, no values were given for phosphatidylinositol. These authors used tritiated water and [1-^{14}C]sodium acetate as precursors, both of which are probably incorporated into the fatty acid moiety of phospholipids. The marked decrease in fatty acid synthesis and in certain enzymes such as acetate thiokinase in sciatic nerve from diabetic animals [11] may, in part, explain these apparently divergent results.

The neuropathies of diabetes and galactosemia are similar in their manifestations and course, both conditions are associated with an elevation of the appropriate blood sugar and tissue content of polyol pathway intermediates, which are believed to cause swelling and osmotic damage [6,12]. There is evidence that elevated levels of glucose and galactose decrease the uptake and increase the efflux of myo-inositol of lens [5]. The present experiments, showing that glucose and galactose have marginal inhibitory effects (12-14%) on the incorporation of [2-^{3}H]myo-inositol into phospholipid, are in keeping with the action of

these sugars on transport processes, however the degree of inhibition is considerably less than that found in the diabetic rat nerve preparations.

The present negative effects of high concentrations of polyhydric alcohols on myo-inositol incorporation into phospholipids of normal sciatic nerve segments, might be due to problems of penetration and uptake into nerve. However, Wells *et al.* [27] and Benjamin & Agranoff [28] have reported no effect of galactitol on phosphoinositide synthesis or on CDP-diacyl glycerol-inositol phosphatidyltransferase (EC 2.7.8.11) of rat brain. Similarly, it has been reported that there is no effect of galactitol on myo-inositol synthesizing enzymes in rat brain [27] and peripheral nerve [29]. It is proposed, therefore, that insulin plays a major role in the uptake of myo-inositol into peripheral nerve and that many of the manifestations of diabetic neuropathy stem from this change.

Myo-inositol is an important factor in phospholipid synthesis and hence of membrane function. Myo-inositol deprivation has been shown to lead to impairment of K^+ and amino acid transport in cell cultures [30] and this may be pertinent in relation to the depression in ^{14}C-leucine incorporation into protein component of myelin in diabetic rat nerve [26].

It has been shown that both di- and tri-phosphoinositides have a high rate of turnover in nerve trunks [31] and that phosphoinositides may be involved in propagation of action potentials and implicated in the control of Ca^{2+} movement at the plasma membrane (see [14]).

Inositol 1-phosphate has been reported to be a cofactor for the enzyme that pyrophosphorylates thiamine [32]. If this occurs in peripheral nervous tissue then the level of inositol 1-phosphate may regulate both pyruvate dehydrogenase and transketolase reactions via thiamine pyrophosphate formation. The di- and triphosphates of thiamine have also been implicated in the slow exponential decline of ionic currents in the nodes of Ranvier [33]. Thus the reported decrease in the level of free myo-inositol and the lowered incorporation of myo-inositol into phospholipid may have far-reaching effects on the physiology, chemistry, structure and function of peripheral nervous tissue.

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