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$ACTH_{4-9}$ analogue ORG 2766 can improve existing neuropathy in streptozocin-induced diabetic rats

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Abstract. The effect of treatment of an existing neuropathy in streptozocin-induced diabetic rats with the $ACTH_{4-9}$ analogue ORG 2766 was examined. Four groups of rats were studied: group 1 consisted of age-matched, non-diabetic controls and groups 2, 3 and 4 of diabetic rats. Sensory and motor nerve conduction velocity (SNCV and MNCV) were measured at weeks 0, 4, 6, 8 and 10. Four weeks after the administration of streptozocin (STZ) all diabetic rats showed a significant slowing of SNCV and MNCV. Treatment was then started: group 2 was treated with placebo, group 3 with a low dos (1 μg) of $ACTH_{4-9}$ subcutaneously every 48 h, and group 4 with a high dos (10 μg) of $ACTH_{4-9}$ subcutaneously every 48 h. The animals treated with the high peptide dosage showed a significant improvement in both SNCV and MNCV from week 6 onwards, whereas this beneficial effect was not demonstrated for the rats treated with the low dosage. This study demonstrates that the $ACTH_{4-9}$ analogue ORG 2766 can ameliorate existing diabetic neuropathy in STZ-induced diabetic rats.

Key words: $ACTH_{4-9}$ – Experimental diabetic neuropathy – Neuropeptide

Introduction

Streptozocin-induced diabetic rats are frequently used to study experimental neuropathy. Various investigators have shown that after induction of diabetes with streptozocin (STZ) different abnormalities occur that reflect neuropathological changes: a reduction in motor nerve conduction velocity and an abnormal, increased resistance to ischaemic conduction blockade [1], morphological changes such as a decrease in axon-to-myelin ratio [2] and biochemical changes, such as myo-inositol depletion and a reduced activity of Na^+-K^+-ATPase [3]. A number of pathogenetic mechanisms have been implicated in the development of experimental diabetic neuropathy (EDN). Finegold et al. [4] have stressed the importance of increased activation of the polyol pathway: chronic hyperglycaemia leads to the conversion of glucose into sorbitol, catalysed by the enzyme aldose reductase. Sorbitol is converted into fructose, and the accumulation of sorbitol and fructose causes a depletion of myo-inositol, resulting in nerve dysfunction possibly by interference with the cellular uptake mechanism for myo-inositol [4].

Low et al. [5, 6] have clearly demonstrated the importance of chronic hypoxia in EDN. They have shown that rats subjected to chronic hypoxia develop electrophysiological abnormalities very similar to those of EDN in the absence of hyperglycaemia, sorbitol accumulation or myo-inositol depletion [7]. In further experiments it was revealed that endoneurial hypoxia can lead to increased oxygen free radical activity and decreased lipid hydroperoxides [8]. Vlassara et al. [9, 10] have stressed that chronic hyperglycaemia, which causes non-enzymic glycosylation of myelin products in the central and peripheral nervous systems, could also be one of the pathogenetic factors of EDN. It has been demonstrated that a decrease in nerve conduction velocity occurs 2–3 weeks after the induction of diabetes with STZ [1]. Hence rats so treated can be used to study diabetic neuropathy by monitoring nerve conduction velocity.

Different approaches have been used in intervention studies related to EDN [3, 11]. A new development has been the use of a neurotrophic analogue of ACTH in EDN. It has been demonstrated that the $ACTH_{4-9}$ analogue ORG 2766 is effective in preventing cisplatinin-induced neuropathy in rats [12, 13] and in patients with ovarian carcinoma [14]. Van de Zee et al. [15] have demonstrated that $ACTH_{4-9}$ is effective in preventing the occurrence of neuropathy in STZ-induced diabetic rats. In this experiment we studied the effect of treatment with $ACTH_{4-9}$ on STZ-induced diabetic rats with an established neuropathy as evidenced by a slowing of nerve conduction velocity. To this end we sequentially measured sensory and motor nerve conduction velocities.

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Materials and methods

Animals

Sixty male rats (mean weight at onset of the study 300 g) of an inbred Wistar strain (–CpB, Toegepast Natuurwetenschappelijk Onderzoek, Zeist, The Netherlands) were studied. All rats were housed in Makrolon cages (two animals per cage) and maintained on a 12:12 h light:dark cycle (lights on at 0730 hours) with normal chow and water ad libitum.

Group 1 consisted of 15 non-diabetic, age-matched controls. Diabetes was induced in 45 rats with a single injection of streptozocin (Zanosar, Upjohn, Kalamazoo, Mich.) 50 mg/kg body weight intravenously given at week 0. After 48 h glucose levels were measured in whole blood obtained from the tail vein with Minilab (Bayer, Munich, Germany); an animal was considered diabetic when the glucose level was above 15 mmol/l. Glucose levels were measured every 2 weeks for the entire study period.

At weeks 0, 4, 6, 8 and 10 sensory and motor nerve conduction velocity (SNCV and MNCV) were measured as described by Bravenboer et al. [16].

After 4 weeks, when a significant neuropathy had developed in the diabetic animals, as shown by a slowing of SNCV and MNCV compared with non-diabetic, age-matched controls, the diabetic animals were divided into three treatment groups: group 2 ($n=15$) was treated with placebo (0.5 ml saline) subcutaneously (s.c.) every 48 h; group 3 ($n=15$) was treated with 1 μg $ACTH_{4-9}$ s.c. every 48 h; and group 4 ($n=15$) with 10 μg $ACTH_{4-9}$ s.c. every 48 h. The investigator performing subsequent measurements had no prior knowledge of the sample identity.

Peptide treatment

ORG 2766, a stable $ACTH_{4-9}$ analogue, was a gift from Organon, Oss, The Netherlands. The peptide was dissolved in 0.5 ml 0.9% saline.

Electrophysiology

The electrophysiological examinations for deterrmination of nerve conduction velocities were carried out under general anaesthesia (Hypnorm, Duphar, Weesp, The Netherlands, containing fluanisone 10 mg/ml and fentanyl citrate 0.2 mg/ml, dose 0.8 mg/kg body weight, administered subcutaneously). To minimize effects of body temperature differences on conduction velocity, the animals were placed under a 40-W light bulb (at a distance of 25 cm) 5 min before testing. During testing, the rectal temperature varied from 37 to 38 °C. The method is described in detail elsewhere [16]. The sciatic and tibial nerves were stimulated at the sciatic notch and ankle, respectively, by means of monopolar needle electrodes. The stimulus was a 500-μs unipolar pulse, generated by a Neurolog NL 300 pulse generator. This stimulus was scaled by a Neurolog NL 510 pulse buffer and delivered by a Neurolog NL stimulus isolator. The stimulation voltage was 42 V; the current was between 1 and 10 mA. The signal was recorded by surface electrodes (Nicolet, 1 mm diameter, 20 Hz–10 kHz) and digitized (Gould Digital Storage Scope). The digitized signal was analysed with specially designed software on a personal computer. The anode was placed 5 mm proximal to the cathode. Two responses can be recorded from the small muscles of the foot by means of surface electrodes after stimulation of these mixed peripheral nerves: the short-latency M-response, due to stimulation of α-motor fibres, and the long-latency H-response, due to stimulation of afferent Ia fibres, which monosynaptically excite α-motoneurons in the spinal cord [17, 18]. The motor nerve conduction velocity (MNCV) and H-reflex-related sensory nerve conduction velocity (SNCV) can be calculated from the latencies of these responses and the distance between the two stimulation points [16, 18].

Table 1. Body weight and glucose levels of groups 1 (controls), 2 (placebo-treated), 3 (1 μg $ACTH_{4-9}$-treated) and 4 (10 μg $ACTH_{4-9}$-treated)

	n	Week 0	Week 4	Week 10	Glucose
Group 1	15	306±9	382±8*	447±10*	6.5±0.5*
Group 2	12	306±9	267±10	265±14	29.0±2.2
Group 3	11	308±11	268±14	274±14	28.7±1.6
Group 4	13	296±10	245±11	241±14	28.9±1.7

Mean weight ±SEM (g) is given per group; whole blood glucose ±SEM (mmol/l) is given as the mean value of measurement 48 h after streptozocin injection and of measurements every 2 weeks onwards

* $P<0.01$ between group 1 and groups 2, 3 and 4

n, Number of rats per group

Statistical analysis

The weights and glucose levels of groups 1, 2, 3 and 4 are given as means ± SD. Student's Newman-Kuels' test was used to compare the means of all groups. Data for the SNCV and MNCV of each group are given as means ± SEM at weeks 0, 4, 6, 8 and 10. At week 4 the treatment code was partly broken to test for a significant difference in SNCV and MNCV between the non-diabetic, age-matched controls and the diabetic animals; Student's *t*-test was used for comparison.

A multivariate analysis of variance with repeated measures (MANOVA) was used to test for differences between groups, to test for time trends and to test for an interaction between groups and time [19]. In group 2 three animals died of causes not related to the diabetic state or anaesthesia; in group 3 four rats died and group 4 two rats. These animals were excluded from the statistical analysis.

Results

Weight and glucose levels

The weight changes and glucose levels of groups 1, 2, 3 and 4 are shown in Table 1. Group 1 (non-diabetic controls, $n=15$) showed a steady increase in weight, from 306±9 g at the beginning of the study to 447±10 g after the 10-week study period. Groups 2 ($n=12$), 3 ($n=11$) and 4 ($n=13$) showed weight losses of 306±9 to 265±14 g, 308±11 to 274±14 and 296±10 to 241±14 g, respectively. The mean weight of group 1 (controls) at weeks 0, 4 and 10 was significantly different from the mean weight of groups 2, 3 and 4. There was no significant difference in mean weight between groups 2, 3 and 4 at weeks 0, 4 and 10.

The mean glucose level (mmol/l) of group 1 was 6.5±0.5, of group 2 29.0±2.2, of group 3 28.7±1.6 and of group 4 28.9±1.7. These values are based on the means of all measurements per group. There was no significant difference between the mean glucose levels of groups 2, 3 and 4.

Nerve conduction velocity studies

The mean SNCV and MNCV (m/s) at weeks 0, 4, 6, 8 and 10 are shown in Figs. 1 and 2, respectively. As expected, group 1 showed an increase in SNCV and MNCV from 49.6±0.6 to 60.2±0.4, and from 49.8±0.8 to

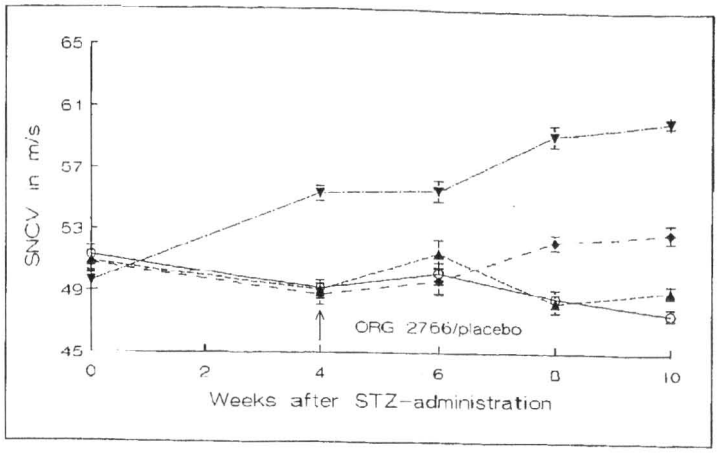

Fig. 1. Sensory nerve conduction velocity (*SNCV*; mean ± SEM) of groups 1 (non-diabetic control animals, $n=15$; ▼), 2 (placebo-treated diabetic animals, $n=12$; ○), 3 (1 μg $ACTH_{4-9}$-treated animals, $n=11$; ▲) and 4 (10 μg $ACTH_{4-9}$-treated animals, $n=13$; ♦) after 0, 4, 6, 8 and 10 weeks. *STZ*, Streptozocin

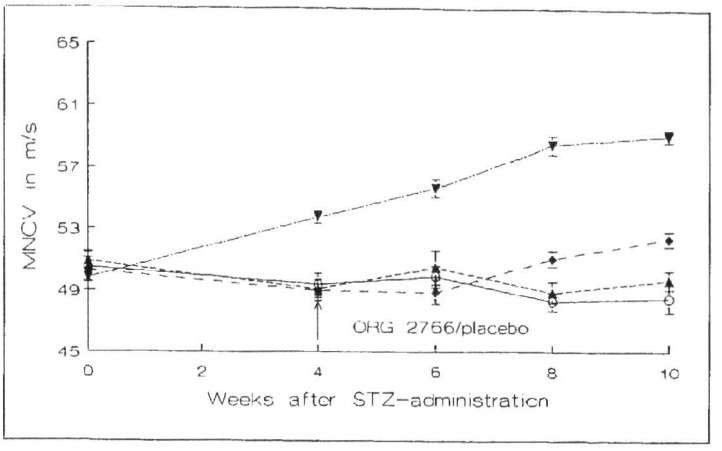

Fig. 2. Motor nerve conduction velocity (*MNCV*; mean ± SEM) of groups 1 (non-diabetic control animals, $n=15$; ▼), 2 (placebo-treated diabetic animals, $n=12$; ○), 3 (1 μg $ACTH_{4-9}$-treated animals, $n=11$; ▲) and 4 (10 μg $ACTH_{4-9}$-treated animals, $n=13$; ♦) after 0, 4, 6, 8 and 10 weeks. *STZ*, Streptozocin

59.1 ± 0.3 m/s, respectively, during the 10-week study period, reflecting normal maturation. In group 2 the SNCV decreased from 49.2 ± 0.5 to 47.6 ± 0.4 and the MNCV from 49.4 ± 0.7 to 48.5 ± 0.9 m/s from week 4 to week 10, respectively. Over the same time period the SNCV changed from 49.1 ± 0.6 to 49.1 ± 0.4 and the MNCV from 50.9 ± 0.6 to 49.7 ± 0.6 in group 3, whereas in group 4 the SNCV increased from 48.8 ± 0.7 to 52.9 ± 0.6 and the MNCV from 49.0 ± 0.7 to 52.4 ± 0.5 m/s.

There was no significant difference between the SNCV and MNCV of groups 2 and 3 from weeks 4 to 10. However, the SNCV of group 4 (diabetic rats treated with 10 μg $ACTH_{4-9}$ was significantly different ($P=0.001$) from that of group 2 (placebo-treated diabetic animals) and group 3 (rats treated with 1 μg $ACTH_{4-9}$) from weeks 4 to 10. The same was true for MNCV ($P=0.023$).

Subanalysis of the treatment effect of group 4 versus that of groups 2 and 3 revealed that the greatest difference in SNCV ($P<0.005$) and MNCV ($P=0.001$) occurred between weeks 6 and 8.

Discussion

It is clear from the electrophysiological data that $ACTH_{4-9}$ is effective in ameliorating an existing diabetic neuropathy in STZ-induced diabetic rats. The dosage of 10 μg $ACTH_{4-9}$ every 48 h is effective, whereas 1 μg is not. This confirms earlier work of Van der Zee et al. [15]. $ACTH_{4-9}$ could not completely restore nerve conduction velocity (NCV) to the same levels as in the non-diabetic controls, probably because of the severity of the hyperglycaemia and the loss of weight in the diabetic animals. Perhaps NCV would have been restored completely if the treatment had been given together with insulin, as has been shown by others [2, 11].

In general there are two possible strategies in the search for a putative therapy in EDN. The first approach takes the specific neuropathology of EDN into account and aims to counteract metabolic abnormalities at different levels in the disease process. For instance, using different putative protective drugs other investigators have obtained similar results in the treatment of EDN. Greene et al. [1] demonstrated that oral administration of myo-inositol can ameliorate the diabetes-induced impairment of MNCV. In 1984 Tomlinson et al. [11] were successful in preventing the development of defects of axonal transport and conduction velocity with an aldose reductase inhibitor. Using protein kinase C agonists in vitro, Kim et al. demonstrated that Na^+-K^+-ATPase activity could be restored in diabetic rats to the same extent as with orally administered myo-inositol [20]. An adequate supply of oxygen can prevent some of the electrophysiological and biochemical abnormalities of EDN [6]. The oral prostaglandin E1 analogue, OP-1206. αCD, ameliorates the decrease in nerve ATPase activity without affecting

nerve myo-inositol levels [21]. This analogue also dramatically improves MNCV in STZ-induced diabetic rats. Recently we were able to show that the free radical scavenger glutathione is beneficial in the prevention of experimental neuropathy [16].

The second approach is based on the theory that neurotrophic factors involved in the differentiation and maturation of nervous tissue may also play a role when that tissue is compromised and in need of repair during adult life. Examples of such an approach are the use of nerve growth factor (NGF) and the $ACTH_{4-9}$ analogue in various models of peripheral nerve diseases [22]. $ACTH_{4-9}$ has been studied extensively as a neurotrophic substance in both the peripheral and central nervous systems. It has been shown to improve functional and electrophysiological parameters of the regenerating sciatic nerve in the rat [23], and to stimulate collateral sprouting in rat soleus muscle after partial denervation [24]. $ACTH_{4-9}$ has also been studied with regard to cisplatinin-induced neuropathy, and is effective in preventing this neuropathy both in rats [12, 13] and in patients with ovarian cancer [14]. Van der Zee et al. [15] were the first to show that $ACTH_{4-9}$ has a beneficial effect in the treatment of peripheral neuropathy in STZ-induced diabetic rats. Recently we reported the efficacy of $ACTH_{4-9}$ in the treatment of diabetic neuropathy occurring in a genetically predisposed diabetic rat strain (*BB/Wor*), in which the diabetic rats received a maintenance dose of insulin [25]. Although collectively the animal data look promising, care is always needed in extrapolating the results of animal experiments to the human condition.

It is obvious from these studies that no single intervention can completely abolish all the electrophysiological, biochemical and morphometric abnormalities that occur in EDN. Combination treatments may increase effectiveness. We conclude that $ACTH_{4-9}$ given at dosage of 10 μg every 48 h is partially effective in ameliorating an existing neuropathy in STZ-induced diabetic rats.

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