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Hypoxic neuropathy versus diabetic neuropathy

An electrophysiological study in rats

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Summary

In the experimental rat model of diabetes a slowing of nerve conduction velocity and a resistance to ischemic conduction failure have been found as an indication of polyneuropathy. The same electrophysiological abnormalities have been demonstrated in a model in which healthy rats are kept under hypoxic conditions (10% O_2) for a 10-week period. Two factors are held responsible for the development of diabetic polyneuropathy: metabolic deterioration and hypoxia. However, until now the relative roles of metabolic deterioration and hypoxia in the development of polyneuropathy have not been settled. To test both explanations further with more sophisticated electrophysiological techniques, the H-reflex (motor and sensory NVC) and the stimulated SF-EMG (measures terminal nerve branch and neuromuscular transmission) were measured in 3 groups of 10 rats, a healthy control group, a diabetic group, and a hypoxic group, every 5 weeks, for 6 months. In the control rats an age-related increase in motor and sensory conduction velocity was found, whereas in the diabetic rats as well as in the hypoxic rats a marked decrease in sensory and a slight decrease in motor nerve conduction velocity was observed. The jitter measured in the stimulated SF-EMG was significantly increased in both the diabetic and the hypoxic group. The results of the present study support the possible role of hypoxia, in addition to metabolic factors, in the development of experimental diabetic neuropathy.

Introduction

Recently, attention has been focused on vascular changes and the role of hypoxia and ischaemia in the pathogenesis of diabetic neuropathy (Low et al. 1984; Dyck 1989). Electrophysiological studies supporting the hypoxia hypothesis have been performed in rats with experimental diabetes (Low et al. 1984; Tuck et al. 1984), in which caudal nerve conduction velocities were measured in the rats' tails (Low and Schmelzer 1983; Low et al. 1984). The most characteristic findings obtained were a slowing of nerve conduction velocity and a resistance to ischemic conduction failure. The initially reduced conduction velocity in the diabetic rats tended to normalize after oxygen supplementation (Low et al. 1984; Low and Tuck 1987). It is unknown which nerve fibre type is most susceptible to the damaging influences of metabolic alterations or hypoxia. Thus experiments in which all fibre types of selective nerves are examined are to be preferred.

Recently, tests have been introduced for the separate examination of peripheral motor and sensory nerves by means of transcutaneous stimulation of the sciatic nerve and recording of H- and M-responses (Stanley 1981; De Koning et al. 1987). With stimulated single fibre EMG (SF-EMG) it is possible to measure neuromuscular jitter, i.e. the variability of intervals between the stimulus and single fibre action potentials during electrical stimulation of motor nerve fibres (Trontelj and Stålberg 1986; Verschuuren et al. 1990). Increased jitter implies imperfect neuromuscular trans-

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mission at immature motor end-plates, which might be an indication of axonal degeneration and regeneration (Stålberg et al. 1975).

The aim of the present study was to gain a better insight into the role of hypoxia as compared with that of diabetes in the development of a polyneuropathy. In both hypoxic and diabetic animals it has been postulated that nerve conduction velocity is reduced by axonal atrophy (Behse et al. 1977; Yagihashi and Matsumaja 1979). By demonstrating neurophysiological changes in hypoxic and diabetic animals with the use of extended and selective techniques, the role of hypoxia and diabetes in the development of neuropathy may be elucidated. Therefore, motor and sensory conduction velocity, as well as motor end-plate function, were investigated in a long-term study in rats with streptozotocin-induced diabetes, chronic hypoxic, and healthy age-matched control animals.

Materials and methods

Animals

Male Wistar rats from an inbred strain (TNO, Zeist, NL), weighing 240–280 g at the onset of the experiment (age 10–12 weeks), were studied.

Three experimental groups were formed. They were matched for age and weight. Group A consisted of 10 healthy non-diabetic, non-hypoxic rats that served as controls and were not treated. Group B consisted of 10 diabetic animals. Diabetes was induced by a single intravenous injection of Streptozotocin (Zanosar®, The Upjohn Company, Kalamazoo, MI 49001, U.S.A.), 50 mg/kg body weight. Group C consisted of 10 rats that were subjected to hypoxia in a low oxygen ($10 \pm 2\%$ O_2) chamber for 6 months. Each group contained 8 extra rats. These received the same treatment as the experimental group but served as blood donors for blood gas analysis to check for hypoxia.

During the experimental period all rats, including the hypoxic rats, were housed and treated in an identical manner: 2 rats per cage and free access to rat chow and water. They were maintained on a 12-h dark-light cycle, with lights on from 06:00 to 18:00 h.

Hypoxic chamber

The hypoxic animal chamber was constructed from iron sheets on 5 sides ($40 \times 50 \times 100$ cm) (Cryer and Bartley 1974). The front side was made of perspex. The seals between the sides were made of rubber. The gas mixture was passed into the chamber via a rubber tubing in the right top corner. The oxygen concentration, temperature and humidity were electronically checked daily. The required oxygen level, range 8–12%, was maintained by administrating a mixture of air and nitrogen, with an inflow of 6 l/min. The gas mixture was brought to ambient temperature by cooling. The chamber contained 6 cages. The hypoxic conditions were interrupted twice a week when food and water were replenished and the cages were cleared. The 10% oxygen level was restored in approximately 40 min.

Metabolic control

One week after the streptozotocin injection, blood glucose levels were determined with Haemo-Glucotest strips (Boehringer®, Mannheim, F.R.G.). Rats displaying glucose levels > 16 mmol/l were considered diabetic (Jakobsen et al. 1987). To check the metabolic state of the three experimental groups, the haemoglobin content of arterial blood was determined and blood gases were analysed on day 2, 8, 28 and 60. Three millilitres of blood was taken by heart puncture and, within 15 min., was analysed in a blood gas analyser (Acid-base laboratory 3 (radiometer ABL 3), Copenhagen).

Electrophysiology

All electrophysiological examinations were carried out under general anaesthesia (Hypnorm®, Duphar, Weesp, containing fluanisone 10 mg/ml and phentanyl citrate 0.2 mg/ml, dose 0.8 ml/kg body weight, administrated subcutaneously). To minimize effects of body temperature differences on conduction velocity, the animals were placed under a 60 W light bulb at a distance of 30 cm. The entire recording and examination procedure lasted approximately 1h. The electrophysiological testing consisted of determination of nerve conduction velocity and stimulated single fibre EMG (stim. SF-EMG). The method for determining sensory and motor nerve conduction velocities has been described in detail by De Koning et al. (1987) (Fig. 1). The tibial and sciatic nerve were stimulated at the ankle and sciatic notch, respectively, using monopolar needle electrodes. The M-response (0.2 ms square wave pulse, supramaximal) and the H-reflex (0.5 ms square wave pulse, submaximal) were recorded from a small muscle on the plantar surface of the hindfoot by means of 5.0 mm surface recording electrodes (Stanley 1981). Two responses occur after stimulation of the mixed tibial or sciatic nerve. Response latencies were defined as the interval between stimulus artefact and the beginning of the deflection of the M- or H-response. The M-response obtained distally and proximally is a direct motor response with a short latency due to stimulation of the α-motor fibers. The motor conduction velocity is calculated as follows: distance sciatic notch-ankle divided by the latency difference M-response sciatic notch-ankle. The H-reflex is an indirect response with a long latency due to stimulation of the afferent proprioceptive Ia-fibers. The H-reflex related sensory conduction velocity is calculated as follows: distance sciatic notch-ankle divided by the latency difference H-re-

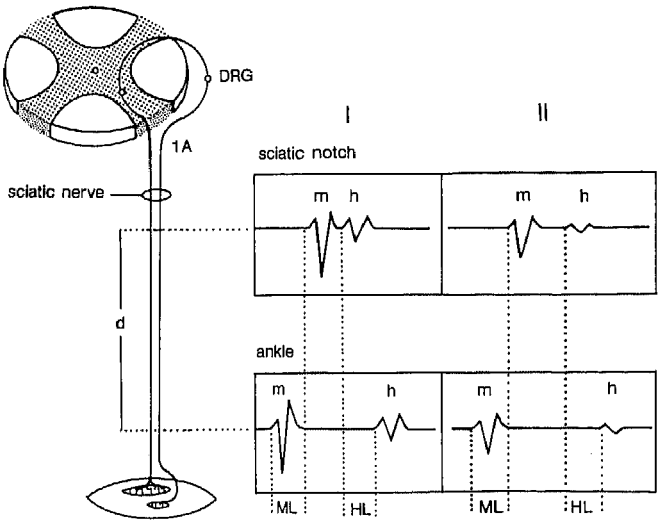

Fig. 1. Illustration of the neurophysiological method used for measuring the motor and sensory NCV. The sciatic nerve is stimulated at the ankle and at the sciatic notch (d = distance between stimulation points). The M-response and the H-reflex are recorded from the plantar muscles of the foot. I = recorded responses at start; II = recorded responses after 6 months in the experimental (hypoxic and diabetic rats) groups. DRG = dorsal root ganglion, α = α-motor neuron, 1A = 1A sensory afferent fiber. MNCV = d/ML; SNCV = d/HL.

sponse ankle-sciatic notch (Stanley 1981; De Koning et al. 1987).

Stimulated single fibre EMG was carried out in the gluteus medius muscle. The method used has been described by Verschuuren et al. (1990). The rats were restrained on a board and the gluteal area was shaved. Intramuscular stimulation was performed proximally in the muscle at the end-plate area, using a monopolar needle with a 0.4 mm bared tip (cathode). The anode was inserted subcutaneously at a distance of about 3 cm. The recording electrode was introduced into the middle part (contracting part) of the muscle. Stimulus frequency: 5 and 10 Hz; stimulus duration: 0.05 ms; stimulus intensity: above threshold value. In each rat 10–20 neuromuscular synapses were studied. SF signals were recorded on a tape recorder (jitter below 4 μs) and analysed on a Dantec Counterpoint apparatus (Fig. 2). Neuromuscular jitter was computed as the mean consecutive difference (MCD) from a series of 50 to 100 time intervals between stimulus artefact and single fibre action potential. To avoid muscle jitter, MCD values less than 8 μs were rejected (Verschuuren et al. 1990).

Experimental design

In the week prior to the start of the actual experiment 10 rats of each group were weighed and examined neurophysiologically (H-reflex and stimulated SF-EMG). The non-treatment animals of group A served as controls. On day 1 group B rats were treated with streptozotocin i.v., and the rats of C group were put in the hypoxic chamber. Every week, six rats were examined, two from each group. During the experimental period all rats were weighed and examined neurophysiologically 5 times, with a fixed time interval of 35 days. The experiment was completed after 6 months. All measurements were carried out by the same investigator.

Data analysis

For data analysis 5 time blocks were formed, block 0 comprises the results of the starting measurements, block 1 the results of the first experimental examination of 10 rats of each group from day 5 to day 39. No explanations could be found for the relatively large standard deviations of H-reflex and SF-EMG at start and in the first block. Therefore, the nerve conduction velocities and MCD data were analysed with an analysis of variance with only the time blocks 2–5 as repeated measure and the group as between-subject factor. In this analysis the variance due to the changes within a time block were considered to be negligible in view of the inter individual variances. For further specification of the effects pairwise *t*-tests were used between the groups in each time block. As level of statistical significance $P < 0.05$ was used.

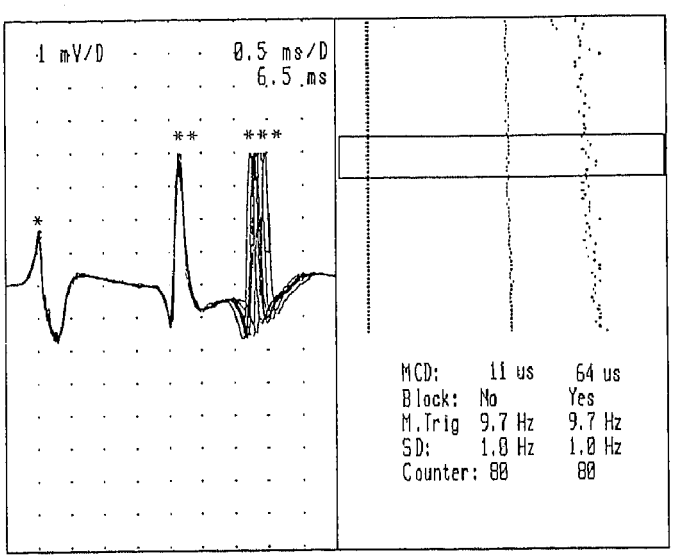

Fig. 2. Recording of jitter measurement in the stimulated SF-EMG. * = stimulation artefact. The recorded potentials as shown on the left side (** and ***) are superimposed visualized. On the right side the analysis of jitter is shown. MCD = mean value of consecutive differences. M.Trig = Stimulation rate. Block = occurrence of impulse blocking yes or no. The MCD value of the first potential (**) is within normal limits, whereas the MCD value of the second potential (***) is increased.

Results

Clinical observations and metabolic control

At the onset of the experiment all rats weighed 269 ± 15.8 g. The mean weight for all groups during the experimental period is shown in Fig. 3. The streptozotocin treated rats developed high glucose levels one week after the i.v. injection. The blood glucose levels increased from 6.7 mmol/l to over 25 mmol/l, and remained elevated throughout the experimental period (Table 1). Streptozotocin-induced diabetes resulted in a marked impairment of growth and weight (Fig. 3). These rats showed muscle wasting, signs of bilateral cataract, and apathy. The hypoxic rats breathing 10% O_2 appeared to have adaptation problems in the first six weeks, resulting in apathy and an impairment of growth and weight (Fig. 3). At the end of the experimental period the hypoxic rats had almost the same weight as the control rats. However, their fur was "fluffy" and they responded aggressively upon handling.

Hypoxia was assessed by arterial blood gas analysis, performed 4 times throughout the experiment (Table 1). The gradual elevation of haemoglobin (and haematocrit) and the increase in blood pH in the hypoxic rats were indicative of adaptation to the hypoxic conditions. The respiratory compensatory mechanisms failed in the diabetic rats, leading to metabolic acidosis.

The experiment started with 3 groups of 10 rats each. All rats survived the 6-month follow-up period. Despite the serious adaptation problems of the hypoxic rats and the progressive wasting and weight loss of the diabetic animals, no animals died in the experimental period.

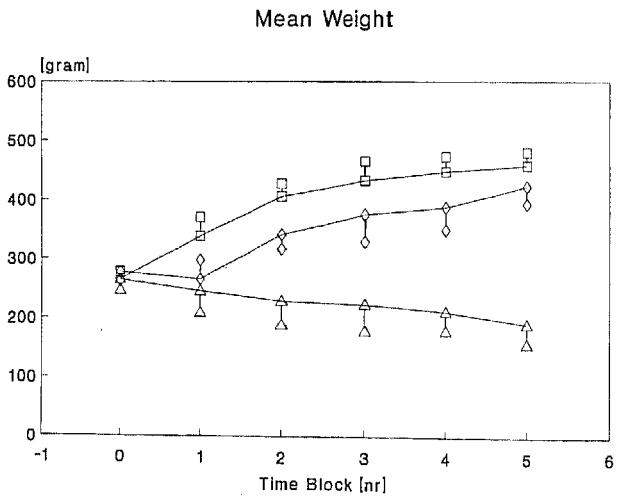

Fig. 3. The weight of the rats during the experimental period. □, Control rats; ◇, hypoxic rats; △, diabetic rats. The values given are the means of 10 rats in one time block + or −1 SD. Block 0 refers to the starting measurements, block 1 the period from day 5 to 39, block 2 day 40 to 74, block 3 day 75 to 109, block 4 day 110 to 144, block 5 day 145 to 180.

TABLE 1

	Day 2	Day 7	Day 28	Day 62
Glucose levels [a]				
Control	6.7	6.7	6.7	6.7
Hypoxic	6.7	6.7	6.7	6.7
Diabetic	> 6.7	> 17.0	25.0	> 25.0 *
Hemoglobin [b]				
Control	15.0	15.1	14.6	14.6
Hypoxic	15.0	16.9	19.8	20.6
Diabetic	15.5	15.0	14.8	14.7
pH [b]				
Control	7.23	7.26	7.28	7.30
Hypoxic	7.18	7.18	7.30	7.34
Diabetic	7.27	7.21	7.18	7.19
Sat% [b]				
Control	83	85	84	92
Hypoxic	30	31	25	25
Diabetic	83	85	89	84

[a] Glucose levels in venous blood, determined with Haemo-Glucotest strips, in the 30 experimental rats (repeating measurements).

* After 62 days glucose levels were determined weekly in diabetic rats only and remained > 25 mmol/l throughout the experimental period.

[b] Blood gas analysis of 3 ml of heparinized arterial blood. The values are the means of two rats for each group.

Electrophysiology

Motor and sensory nerve conduction velocities measured in the three groups during the experimental period are shown in Fig. 4A,B. Age-matched non-diabetic control rats ($n = 10$) showed an increase in motor conduction velocity (MNCV) as well as in sensory nerve conduction velocity (SNCV). The MNCV increased from 51.59 ± 7.09 to 60.59 ± 2.16 m/s, and the SNCV increased from 65.58 ± 4.44 to 69.49 ± 5.04 m/s. However, in the diabetic rats conduction velocities gradually fell below the starting values. In the diabetic rats MNCV decreased from 49.57 ± 10.21 to 37.72 ± 2.39 m/s, and the SNCV decreased from 69.61 ± 4.20 to 43.52 ± 2.64 m/s. The hypoxic rats showed an MNCV at the start of the experiment of 49.02 ± 4.17 and 49.34 ± 2.86 m/s in the 5th time block. The SNCV in this group decreased from 69.92 ± 5.89 to 58.63 ± 3.87 m/s. When comparing the MNCV changes between the three experimental groups, differences between the control group and the diabetic group reached significance in the first time block ($P < 0.001$), between the control and hypoxic group in the second time block ($P < 0.003$), and between the diabetic and hypoxic group in the third time block ($P < 0.001$). The significance of differences between the groups for the SNCV were the same as for the MNCV.

The mean jitter values for each group in the 5 time blocks are shown in Fig. 4C. At start of the experiment the mean MCD value of the gluteus medius muscle of 30 rats was 12.5 ± 1.4 μs (mean ± SD). Range of jitter values in individual muscles was 8–20 μs. In the control group the mean values did not change significantly during the experimental period. After 6 months the mean MCD value in the control group was 11.7 ± 0.3 μs. MCD values in the hypoxic and diabetic groups increased gradually over the 6-month period. At the end of the experiment the mean MCD value in the hypoxic group ($n = 10$) was 16.9 ± 0.6 μs and in the diabetic group ($n = 10$) 17.5 ± 0.5 μs. In comparison with the control group, the differences reached significance in the first time block for the hypoxic animals ($P < 0.004$) and in the second time block for the diabetic rats ($P < 0.001$). The increase in MCD values of the diabetic group were more pronounced and significantly different in comparison with those of the hypoxic group in the third and fourth time block, whereas in the fifth time block differences between the mean jitter values were no longer significant ($P = 0.028$). All comparisons in an analysis of variance with the time blocks 2–5 as repeated measures and the group as between-subject factor were statistically significant ($P < 0.001$).

Discussion

Two theories have been proposed to explain the aetiology of diabetic neuropathy: the metabolic and the vascular theory (Schmidt et al. 1975; Brismar and Sima 1981; Greene and Winegrad 1981; Brismar 1983;

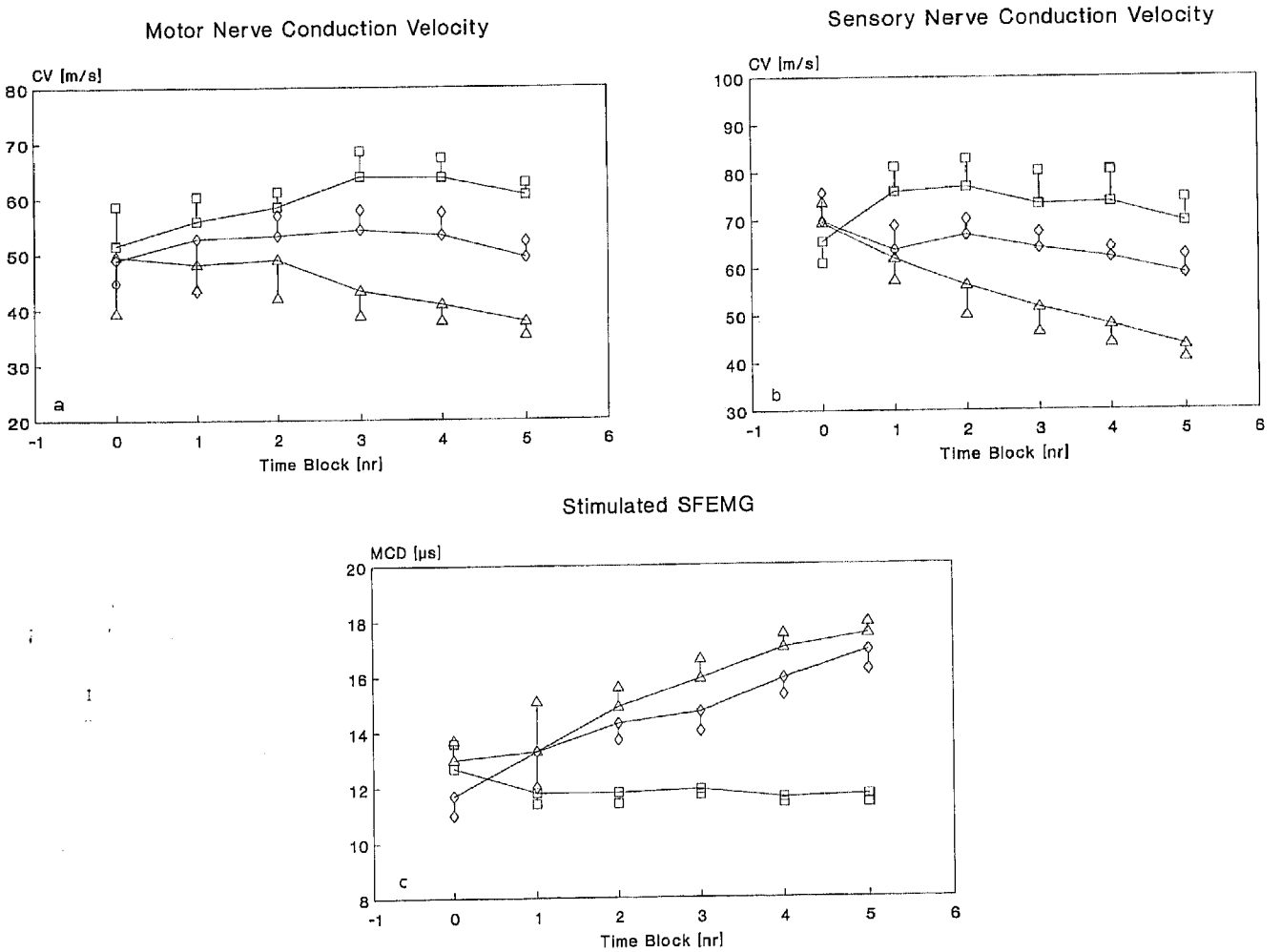

Fig. 4. The longitudinal assessment of motor nerve conduction velocity (MNCV) (A), sensory nerve conduction velocity (SNCV) (B), and the mean consecutive difference (MCD) (C) in the two experimental groups and the controls. The values given are the means of 10 rats in one time block + or −1 SD. See Fig. 3 for time blocks and symbols used for animal groups. Differences in CV (motor and sensory) were significantly different between the three groups after the second time block ($P < 0.001$). MCD values were significantly different between control and experimental groups ($P < 0.001$) after the first time block. MCD values of hypoxic and diabetic rats were only significantly different in the time blocks 2, 3 and 4.

Greene 1984; Low et al. 1984; Thomas and Ochoa 1984; Greene et al. 1985; 1987; Sima and Brismar 1985; Brismar et al. 1987; Low 1987; Dyck 1989). In the vascular theory, nerve ischaemia and hypoxia are thought to be of major importance in the development of the pathological alterations of diabetic neuropathy. Morphological studies on sural nerve biopsies of diabetic patients have shown endoneurial vascular abnormalities (Williams et al. 1980; Dyck et al. 1985; 1986; Britland et al. 1990), as well as axonal degeneration and fibre loss (Behse et al. 1977; Kimura et al. 1979; Yagihashi and Matsumaja 1979; Sima et al. 1988). It has been suggested that this axonal degeneration due to endoneurial hypoxia is the primary event in diabetic polyneuropathy. Evidence in support of the hypoxia hypothesis has been obtained in several human studies as well as in animal models (Korthals and Wisniewski 1975; Schmidt et al. 1975; Korthals et al. 1978; Low et al. 1984; 1986; Dyck et al. 1986; Nukada and Dyck 1987). In an attempt to determine to what extent hypoxia may cause electrophysiological abnormalities in an animal model, Low et al. (1986) submitted normal rats to chronic hypoxia. Rats breathing 10% O_2 had a 56% reduction in nerve blood flow and 40–50% of normal endoneurial oxygen values, values slightly lower than those found in chronic experimental diabetic rats (Tuck et al. 1984). These abnormalities occurred in the absence of severe metabolic alterations such as hyperglycemia, nerve sorbitol accumulation or myo-inositol reduction (Low et al. 1985). However, the mechanism underlying the slowing of nerve conduction remains obscure. A slowing of caudal nerve conduction velocity has been found after 4 weeks of hypoxia (Low and Tuck 1987). In all of these studies, the hypoxic rat model as well as the experimental diabetes rat model examined for a relatively short period of 10 weeks.

In the present study, the hypoxic rats had severe adaptation problems in the first 6 weeks, whereas in the diabetes group the signs of illness and the progressive metabolic dysregulation became evident after 6 weeks. To ensure the reliability of the comparison of the electrophysiological results, an experimental period of 6 month was chosen. The methods used allowed the selective and longitudinal assessment of proximal and distal nerve function as well as motor end-plate function in three experimental groups: healthy control animals, chemically induced diabetic rats, and rats living under hypoxic conditions.

In non-diabetic, non-hypoxic rats, only a small age-related increase in motor and sensory conduction velocity was found, as has been described by other authors (Stanley 1981; De Koning and Gispen 1987; Van der Zee 1989). In diabetic rats, however, the motor conduction velocity did not increase when compared with the values of the control group, indicating a relative decrease. The sensory conduction velocity decreased markedly. The hypoxic rats showed electrophysiological abnormalities similar to those seen in the experimental diabetes group. The findings indicate that hypoxia per se causes a slowing of nerve conduction. Unfortunately, the extent of nerve damage is not known nor the minimal oxygen concentration needed to induce these nerve alterations. The value of 10% O_2 was chosen in agreement with former studies (Kentera and Zdravkovic 1976; Emery 1981; Susic 1981). This value is the lowest that rats can tolerate chronically, without pulmonary hypertension due to anatomical changes in the heart and lungs. However, the 10% environmental O_2 does not guarantee a 50% reduction of endoneurial oxygen values. The actual endoneurial oxygen tension in these experimental rats was probably much higher because the hypoxic animals adapted to the hypoxic conditions, as indicated by the blood gas analysis in the longitudinal assessment.

It should be emphasized that diabetic neuropathy is thought to be the result of hypoxia as well as metabolic alterations. The finding that hypoxia, without these severe metabolic disturbances, had the same effect on thick myelinated rat nerves as a combination of hypoxia and metabolic alterations was an important observation in support of the assumed primary and main role of hypoxia in the development of diabetic neuropathy.

A method to quantify axonal disturbances is the stimulated single fibre EMG (stim. SF-EMG). SF-EMG performed in human diabetic subjects without clinical or electrophysiological evidence of polyneuropathy has shown an increased jitter (Trontelj and Stålberg 1986; Shields 1987), which was suggested to be the result of axonal derangement. The stimulated SF-EMG has proved to be of great value in detecting early motor end-plate dysfunction in animal models (Verschuuren et al. 1990).

In the present study, the jitter values of the control group were in the same range as those reported by Verschuuren et al. (1990). In both the diabetic group and the hypoxic group, an increase in jitter was found. It was assumed that this increase in jitter could not be attributed to changes in the muscles or other variations in structural characteristics of the postsynaptic area, because the muscle conduction velocities in the gluteus medius muscle remained unchanged during the experimental period in the three groups. Measurement of propagation along the muscle fibres confirmed these findings (Hendriksen et al. 1992). Thus stimulated SF-EMG is a sensitive means of detecting the involvement of peripheral nerve in induced diabetic as well as in hypoxic polyneuropathy. It is known that a primary demyelinating process does not impair motor end-plate function (Stålberg and Trontelj 1979). In this situation nerve conduction velocity may be decreased, but the jitter remains within the normal range. When jitter is

increased in combination with a slowing of nerve conduction velocity, as found in the present study in both diabetic and hypoxic animals, it is likely that there is primary axonal degeneration (Shields 1987).

By using sophisticated techniques in matched groups, we could compare the electrophysiological results of the two experimental groups. The curves for the slowing of motor and sensory nerve conduction velocity of the two experimental groups ran parallel but at different levels, indicating differences in the effect of damaging factors on these thick myelinated nerve fibres but also showing their mutual vulnerability. The curves of the MCD values were almost equal. This may indicate that the alterations of terminal branches were mainly influenced by hypoxia whereas metabolic alterations were of minor importance at this level. The findings of the present study support the hypothesis that long-standing hypoxia per se can induce severe alterations in peripheral nerves. Although this electrophysiological finding argues in favour of the assumed importance of hypoxia as a major factor in the development of diabetic polyneuropathy, the problem whether microvascular alterations and hypoxia induce neuropathic abnormalities primarily, or whether metabolic alterations are of main importance in the development of axonal degeneration, remains unsolved.

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