Effects of the Peptide HP228 on Nerve Disorders in Diabetic Rats

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Motor and sensory nerve conduction velocities (MNCV and SNCV) were reduced in the sciatic nerve of rats after 4 weeks of untreated streptozotocin-induced diabetes, and declined further during the following 4 weeks. Treating diabetic rats with the novel peptide HP228 had no effect on the decline of MNCV after the first 4 weeks of diabetes but attenuated the decline in SNCV. HP228 treatment also prevented any further decline in MNCV or SNCV between weeks 4 and 8 of diabetes. Consequently, at the conclusion of the study, the nerve conduction velocities (NCVs) in treated rats were significantly (both $P < .001$) higher than in untreated diabetic rats. Reduced nerve homogenate Na^+,K^+-adenosine triphosphatase (ATPase) activity in diabetic rats was significantly ($P < .05$) increased by HP228 but remained significantly ($P < .05$) lower than in untreated controls. HP228 treatment also reduced nerve Na^+,K^+-ATPase activity of control rats compared with untreated controls ($P < .05$). There was no effect of HP228 on the hyperglycemia, nerve polyol accumulation, *myo*-inositol depletion, reduced nerve laser Doppler blood flow, thermal hypoalgesia, or reduced mean axonal caliber in diabetic rats or on any of these parameters in control rats. These data demonstrate that a novel peptide may protect against the slowing of nerve conduction in prolonged diabetes and that the mechanism of action is unrelated to aldose reductase inhibition, prevention of nerve ischemia, or axonal atrophy. HP228 may prove a potential therapeutic agent for the treatment of prolonged diabetic neuropathy.

NEUROPATHY is the most common complication associated with diabetes mellitus, with nerve dysfunction detectable in as many as one third of newly diagnosed diabetics[1] and more than half of a cohort of patients evaluated for 25 years.[2] The most common presentation is distal symmetric polyneuropathy, and nerve disorders occurring early in the course of neuropathy include slowed motor (MNCV) and sensory (SNCV) nerve conduction velocity,[3,4] resistance to ischemic conduction block,[5] and increased vibration, thermal, and thermal pain perception thresholds.[6,7] Nerve conduction studies are commonly used to measure nerve dysfunction in diabetes, and slowed nerve conduction velocities (NCVs) are considered predictive of progression to overt neuropathy[8] characterized by structural damage to both axons and Schwann cells.

Experimentally diabetic rats develop a number of nerve disorders, including slowed MNCV and SNCV,[9,10] resistance to ischemic conduction block,[11] and thermal hypoalgesia.[12] Although structural changes are not obvious during the initial weeks of diabetes when conduction slowing is first noted and there is no loss of myelinated fibers, a reduction in mean axonal caliber occurs after longer periods of hyperglycemia and may contribute to progressive conduction slowing.[13] Diabetic rats therefore serve as a model for the aspects of nerve dysfunction found in relatively short-term diabetic patients. There have been many reports that the slowing of NCV in short-term diabetic rats may be prevented and reversed by aldose reductase inhibitors (ARIs), implicating an exaggerated metabolism of glucose by the polyol pathway in the etiology of this disorder.[14,15] A variety of agents with the common ability to increase blood flow and prevent oxidative stress also attenuate nerve conduction disorders in diabetic rats.[16] These findings provide support for the suggestion that reduced nerve blood flow[17] contributes to conduction slowing. Studies showing that reduced nerve blood flow in diabetic rats may be prevented by ARI treatment[18-20] suggest that a common mechanism may underlie the efficacy of ARIs and vasoactive agents in early nerve conduction deficits.

It is becoming increasingly apparent that adult peripheral nerves receive ongoing neurotrophic support.[21] A number of reports indicate a diminished neurotrophic support to the peripheral nerve of diabetic rats, with reduced levels of mRNA, protein, or bioactivity of a range of factors including nerve growth factor,[22-24] neurotrophin (NT)-3, NT-4,[25] ciliary neurotrophic factor,[26] and insulin-like growth factor[27] in the target organs or the nerve itself. This has prompted speculation that impaired neurotrophic support also contributes to aspects of nerve dysfunction in diabetic rats and that agents with neurotrophic properties may prove potential therapeutic agents for the treatment of diabetic neuropathy.[28] In the present study, we examined the effect of the novel peptide HP228, an amino acid sequence that resembles fragments of the neuroactive peptides corticotropin (ACTH) and α-melanocyte-stimulating horomone (α-MSH), on nerve dysfunction in diabetic rats.

MATERIALS AND METHODS

Animals and Experimental Design

Adult female Sprague-Dawley rats (Harlan, San Diego, CA) were assigned at random to one of four groups. Diabetes was induced in two groups of rats by a single intraperitoneal injection of streptozotocin (50 mg/kg in 0.9% sterile saline) after an overnight fast. Hyperglycemia was confirmed 2 days later by measurement of the tail vein blood glucose concentration using a glucose oxidase–impregnated test strip (Ames Glucostix; Myles, Elkhart, IN). Two other groups of rats served as age-matched controls. On the day that diabetes was confirmed, one group of control and one group of diabetic rats began a treatment regimen that consisted of daily intraperitoneal injections of 10 μg HP228 (Ac-Nle-Gln-His-(d)-Phe-Arg-(d)-Trp-Gly-NH_2; Trega Biosciences, San Diego, CA) in sterile 0.9% saline. All animals were allowed

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free access to food and water and were maintained under standard conditions.

Thermal Nociceptive Testing

To determine the effect of hyperglycemia on responses to acute thermal nociceptive stimuli, rats were placed in an open-top Plexiglas cylinder on top of a Paw Thermal Stimulation System (UARD, San Diego, CA) with a surface temperature of 30°C. A mobile radiant heat source was maneuvered below the plantar surface of the hindpaw, and heat was applied until the paw was moved. The latency to response was recorded automatically by movement sensors. Responses from both hindpaws were measured 5 minutes apart, and the mean value for both paws was used for analysis.

Nerve Conduction Studies

Nerve conduction studies were performed on all animals before induction of diabetes and also 4 and 8 weeks after the onset of diabetes. Rats were anesthetized with halothane (4% in O_2 for induction and 2% for maintenance), and a thermistor probe was placed adjacent to the right sciatic nerve via a skin incision and blunt separation of the connective tissue fascia between the biceps femoris and gluteus maximus muscles. The incision was closed, and the local temperature was kept at 37°C using a heat lamp and temperature controller during stimulation of the sciatic nerve (5V, 0.05-millisecond single square-wave pulses) at the sciatic notch or ankle. Evoked responses were recorded with needle electrodes placed in the interosseous muscles of the ipsilateral foot, amplified (×100) with a P15 AC Amplifier (Grass Instruments, Quincy, MA), and displayed on a 5110 Storage Oscilloscope and 5D10 Waveform Digitizer (Tektronix, Beaverton, OR). The difference in the response latency of the M-wave following stimulation at the two sites was taken as the time required for motor nerve conduction to proceed from notch to ankle, and the difference in H-wave latency as the time required for sensory conduction from ankle to notch. This procedure was repeated three times for each rat, and the median latency differences were used for calculating conduction velocities. NCVs were calculated by dividing the distance between the stimulation sites by the latency difference.

Nerve Laser Doppler Flux

At the conclusion of the study, the animals were anesthetized with an intraperitoneal injection (2 mL/kg) of a solution consisting of pentobarbital (12.5 mg/mL) and diazepam (1.25 mg/mL) in 0.9% sterile saline. The core temperature was maintained at 37°C using a heating pad, rectal probe, and temperature controller. The mean arterial pressure was measured with a MacLab/8s system (AD Instruments, Castle Hill, Australia) and transducer amplifier after cannulation of the right femoral artery. The left sciatic nerve was exposed as already described, and a pool of mineral oil was applied over the exposed tissues. Temperature readings were made with a thermistor probe placed in the mineral pool close to the sciatic nerve, and the temperature was stabilized at 32° to 33°C. A laser Doppler probe (0.85-mm diameter attached to TSI model Blood Perfusion Monitor 403A, St Paul, MN) was positioned with a micromanipulator above the surface of the midthigh region of the sciatic nerve. Ambient lighting was kept constant, and five measurements were made at 1-mm increments along the nerve when a stable temperature was reached. Laser Doppler flowmetry was chosen because the technique readily yields measurements that are proportional to nerve blood flow without the prolonged anesthesia or surgery required by other methods that measure nerve blood flow. Measurements of whole fascicle flow were favored by the 1-mm tissue penetration depth of the probe and avoidance of large and/or obvious epineurial vessels. Since nerve blood flow values vary according to arterial pressure, laser Doppler flow was standardized to femoral arterial pressure at each time point as the laser Doppler vascular conductance (LDVC) in arbitrary Doppler flow units per millimeter of mercury. The median of the five measurements was used to represent nerve LDVC.

Assays

Following measurement of nerve laser Doppler flux, blood samples were collected from the femoral artery for determination of the plasma glucose concentration by spectrophotometric assay (Glucose Assay Kit; Sigma, St Louis, MO), and the rats were killed by infusion of saturated KCl. Segments of the left sciatic nerve were removed and stored at −70°C until subsequent assay. Nerve water content was calculated as the difference between wet weight and dry weight after freeze-drying for 24 hours. Nerve sugar and polyol levels were measured by assaying their trimethylsilyl derivatives using a Hewlett Packard (Avondale, PA) 5890 gas chromatograph fitted with a 25-m × 0.2-mm Hewlett Packard Ultra 1 capillary column and flame ionization detector with α-methylmannoside included as an internal standard. Water, sugar, and polyol levels were referenced to the nerve dry weight. Na^+, K^+-adenosine triphosphatase (ATPase) activity was measured in homogenates of sciatic nerve as the fraction of total ATPase activity inhibited by 5 mmol/L ouabain.[29]

Morphometry

A portion of the right sciatic nerve was fixed overnight at 4°C in 2.5% phosphate-buffered glutaraldehyde, postfixed in 1% aqueous osmium tetroxide for 3 to 4 hours, and dehydrated using a series of graded alcohols and propylene oxide. After infiltration with a 1:1 mixture of propylene oxide and araldite for 4 hours, nerves were placed in 100% araldite overnight before embedding in fresh araldite resin. Transverse sections (1 μm) were stained with *p*-phenylenediamine before light-microscopic examination and computer-assisted analysis of myelinated fiber size-frequency distributions as described in detail previously.[30] Video images were obtained with an Olympus (Tokyo, Japan) BH-2 light microscope and attached television camera interfaced with a Macintosh (Apple, Seattle, WA) Quadra 850AV computer running National Institutes of Health (Bethesda, MD) Image 1.55 software. Axonal areas surrounded by myelin sheaths of myelinated fibers greater than 1 μm in diameter were identified by eye, and the axonal area was recorded automatically; 3,000 myelinated fibers were examined in each nerve, representing about 60% of the total number of fibers present.

Statistical Analysis

All measurements were performed with coded animals to avoid the possibility of bias. Statistical analyses were performed by one-factor ANOVA, and individual post hoc comparisons were made with the Newman-Keuls method when the F ratio indicated a P value less than .05. The data are presented as the mean ± SEM.

RESULTS

Diabetic rats were hyperglycemic and exhibited reduced body weight compared with the controls (Table 1). Glucose, sorbitol, and fructose accumulated in the sciatic nerve of diabetic rats, while nerve *myo*-inositol levels were depleted compared with the controls (Table 1). Nerve water content was unaffected by diabetes. HP228 had no effect on any of these parameters in control or diabetic rats. Similarly, HP228 did not affect the nerve vascular conductance in control rats or the reduction in vascular conductance ($P < .001$) observed in diabetic rats (Table 2).

HP228 had no effect on thermal response latencies in control rats and did not prevent the thermal hypoalgesia present after 8 weeks of diabetes (Fig 1). Both MNCV (Fig 2) and SNCV

Table 1. Body Weight, Plasma Glucose, and Nerve Sugars and Polyols in Control and 8-Week Diabetic Rats (mean ± SEM)

Group	No.	Body Weight (g)	Plasma Glucose (mmol/L)	Nerve Water (mg/mg)	Nerve Sugars and Polyols (nmol/mg dry weight)			
					Glucose	Sorbitol	Fructose	*myo*-Inositol
Control	10	254 ± 3[a]	5.4 ± 0.4[a]	1.69 ± 0.05	4.15 ± 1.01[a]	0.00	1.47 ± 0.36[a]	11.79 ± 0.41[a]
Control + HP228	10	249 ± 3[a]	5.8 ± 0.3[a]	1.66 ± 0.05	4.77 ± 0.86[a]	0.00	0.94 ± 0.36[a]	11.41 ± 0.31[a]
Diabetic	11	183 ± 7[b]	39.1 ± 2.7[b]	1.70 ± 0.05	45.70 ± 3.18[b]	4.74 ± 0.58	22.38 ± 1.19[b]	7.22 ± 0.20[b]
Diabetic + HP228	10	187 ± 8[b]	44.6 ± 1.7[b]	1.71 ± 0.05	44.77 ± 3.79[b]	4.86 ± 0.51	20.25 ± 1.11[b]	7.26 ± 0.45[b]

NOTE. Statistical comparisons by 1-way ANOVA followed by Newman-Keuls post hoc test: a *v* b, $P < .001$.

(Fig 3) in control rats remained stable during the 8 weeks of the study and were unaffected by HP228 treatment. Untreated diabetic rats showed a decline from the onset values for both MNCV and SNCV such that both were significantly ($P < .001$) lower than in untreated controls after 4 weeks of diabetes and lower still after 8 weeks of diabetes ($P < .001$). HP228 had no effect on the decline of MNCV in diabetic rats measured after 4 weeks of diabetes, but prevented any further decline such that after 8 weeks MNCV was significantly ($P < .001$) higher than in untreated diabetic rats while remaining significantly ($P < .01$) lower than in controls. SNCV of HP228-treated diabetic rats was attenuated after 4 weeks of treatment, and by 8 weeks, it was significantly higher than in untreated diabetics ($P < .001$) but still significantly lower than in untreated controls ($P < .05$).

The mean axonal caliber of the sciatic nerve was lower in untreated diabetic rats (3.75 ± 0.15 μm, n = 6) than in untreated controls (4.08 ± 0.07 μm, n = 6), and there was no effect of HP228 on the mean axonal caliber of control rats (4.07 ± 0.05 μm, n = 7) or the reduced mean axonal caliber of diabetic rats (3.84 ± 0.08 μm, n = 6). Because our measurements of NCV were made in the fastest and therefore largest fibers, we calculated the proportion of fibers with a caliber of 7 μm or greater (Fig 4). Diabetes significantly ($P < .05$) reduced the proportion of large-caliber fibers compared with that of the untreated controls, and HP228 was without effect on the caliber of either control or diabetic nerves.

The reduced nerve homogenate Na^+,K^+ ATPase activity of untreated diabetic rats ($P < .001$ *v* untreated controls; Fig 5), was significantly ($P < .05$) increased by HP228, although it remained significantly ($P < .05$) lower than untreated controls. HP228 also reduced nerve homogenate Na^+, K^+ ATPase activity of control animals ($P < .05$ *v* untreated controls) to levels that were not different from HP228-treated diabetics but significantly ($P < .05$) higher than untreated diabetics.

DISCUSSION

Slowed NCVs develop within 2 to 3 weeks of the onset of hyperglycemia in diabetic rats.[14] Our present findings agree with previous time-course studies that identified a decline in velocity from the onset values in adult diabetic rats that becomes progressively worse over time.[14] We also identified a number of disorders in the nerve of diabetic rats that coincided with reduced NCV. These included accumulation of polyol pathway metabolites, *myo*-inositol depletion, reduced nerve homogenate Na^+,K^+-ATPase activity, decreased nerve blood flow, and reduced mean axonal caliber. All of these disorders have been suggested to contribute to the slowing of NCV.[9,13,14,17,31] The precise mechanisms underlying the conduction slowing in mature diabetic animals are unresolved but are unlikely to arise from either nerve dehydration or edema, since there was no change in the nerve water content of diabetic rats compared with controls.

HP228, a peptide fragment related to ACTH and α-MSH, attenuated the decline in both MNCV and SNCV of mature diabetic rats, notably between weeks 4 and 8 of diabetes. Because our method of measuring NCV records the velocity of the fastest fibers, this suggests an effect on large-fiber dysfunction. In contrast, there was no effect on thermal hypoalgesia in diabetic rats measured at the conclusion of the study, although we did not take measurements at earlier time points and cannot exclude the possibility that HP228 delayed the onset of thermal hypoalgesia. The thermal test measures the responsiveness of unmyelinated and small myelinated sensory fibers that are sensitive to noxious thermal stimuli. Thus, the therapeutic effects of HP228 were evident only on large-fiber function, although a corresponding effect on sensory fibers of other modalities or the NCV of thermosensitive fibers cannot be discounted.

Treating diabetic rats with HP228 did not affect many of the biochemical and physiologic disorders considered to underlie NCV slowing. It did not affect the severity of diabetes, as reflected in the loss of body weight and increased plasma and nerve glucose levels, nor did it act as an ARI, as nerve accumulation of sorbitol and fructose remained high in HP228-treated diabetic rats and *myo*-inositol depletion persisted. Moreover, there was also no effect on vascular conductance, as measured by laser Doppler flowmetry, suggesting no amelioration of the reduced nerve blood flow in diabetic rats. Laser

Table 2. Cardiovascular Parameters and Nerve Laser Doppler Flux in Control and 8-Week Diabetic Rats (mean ± SEM)

Group	No.	Blood Pressure (mm Hg)	Heart Rate (bpm)	Nerve Flux (U)	Nerve Conductance (U/mm Hg)
Control	10	111 ± 2[a]	347 ± 10[a]	51.9 ± 5.6[a]	0.46 ± 0.05[a]
Control + HP228	9	113 ± 3[a]	352 ± 11[a]	54.4 ± 3.6[a]	0.48 ± 0.03[a]
Diabetic	10	95 ± 5[b]	299 ± 6[b]	21.0 ± 2.7[b]	0.22 ± 0.02[b]
Diabetic + HP228	9	104 ± 4	322 ± 9	26.1 ± 3.5[b]	0.25 ± 0.03[b]
		a *v* b, $P < .01$	a *v* b, $P < .01$	a *v* b, $P < .001$	a *v* b, $P < .001$

NOTE. Statistical comparisons by 1-way ANOVA followed by Newman-Keuls post hoc test.

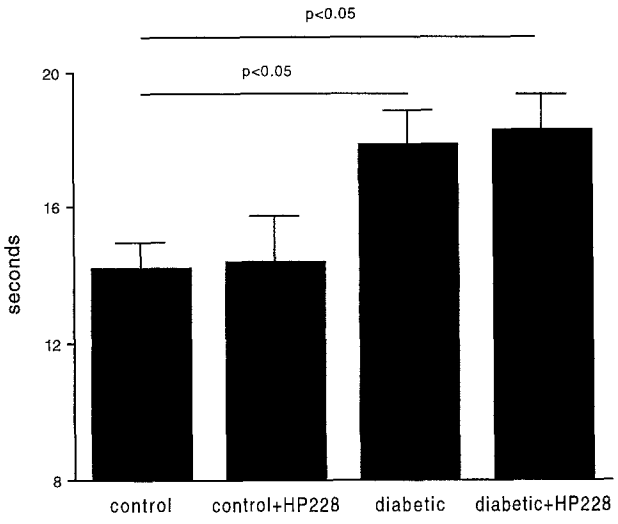

Fig 1. Thermal response latencies in the hindpaw of control and diabetic rats with or without treatment with HP288. Data are the mean ± SEM; n = 9 to 11.

Doppler flowmetry measures the flux in vessels of both the endoneurium and epineurium, and the relative decrease in flow (approximately 50%) is similar to that reported in the endoneurium by other techniques.[17,32,33] While it is plausible that a measurement of increased nerve laser Doppler flux might not necessarily reflect any increase in endoneurial blood flow, due to selective epineurial vessel shunting,[34] the converse, namely an increase in endoneurial flow being undetected by laser Doppler flowmetry, appears unlikely. Indeed, increases in the endoneurial blood flow of diabetic rats caused by treatment with ARIs,[20] evening primrose oil,[35] angiotensin receptor antagonists,[36] antioxidants,[37] and endothelin receptor antagonists[38] have previously also been detected using laser Doppler flowmetry.[19,20,39-42] It therefore appears that HP228 exerts protective effects on nerve conduction in diabetic rats without acting via either aldose reductase inhibition or vascular perfusion improvement.

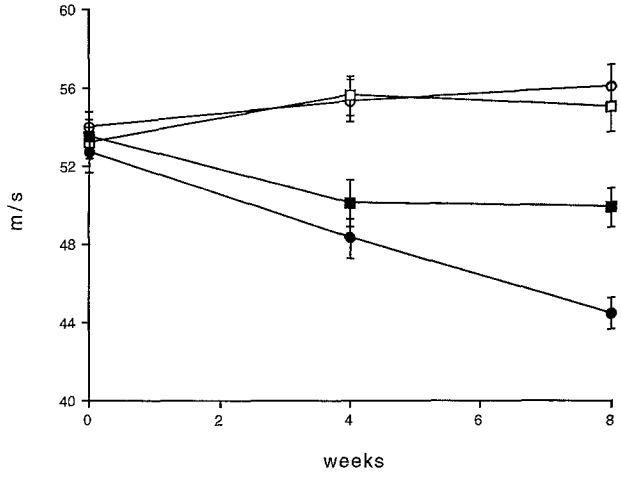

Fig 2. Sciatic (M-wave) MNCV in control (○), control + HP228 (▨), diabetic (●), and diabetic + HP228 (■) rats. Data are the mean ± SEM; n = 9 to 11.

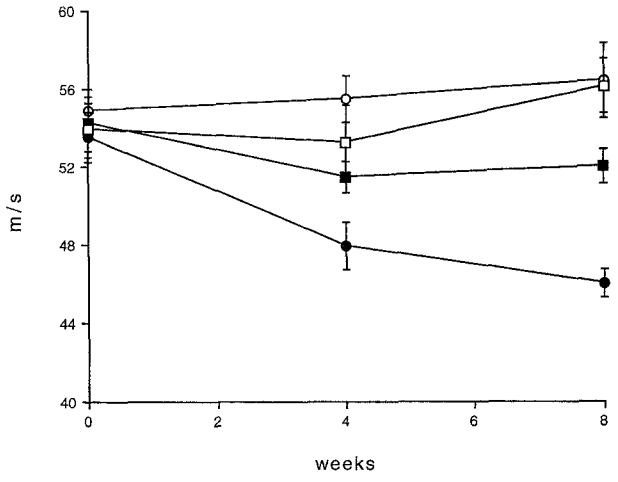

Fig 3. Sciatic (H-wave) SNCV in control (○), control + HP228 (▨), diabetic (●), and diabetic + HP228 (■) rats. Data are the mean ± SEM; n = 9 to 11.

Of the biochemical disorders of diabetic nerve that coexisted with the decline in NCV, the decrease in homogenate Na^+,K^+-ATPase activity was partially improved by HP228 treatment in parallel with the attenuation of conduction slowing. This is consistent with a variety of other agents that improve both homogenate Na^+,K^+-ATPase activity and NCV in diabetic rats and which provide support for the hypothesis that deficient axonal Na^+,K^+-ATPase activity may contribute to NCV slowing[43] by decreasing nodal Na^+ gradients and thereby impeding inward ion flux during impulse propagation.[44] However, a simple association between nerve homogenate Na^+,K^+-ATPase activity and NCV is not supported by our finding that HP228 reduced the Na^+, K^+-ATPase activity of control nerve homogenates but not the NCV. Indeed, a number of studies have dissociated nerve Na^+, K^+-ATPase activity and NCV during hyperglycemia.[29,45-49] Because a range of cells in the nerve trunk possess Na^+,K^+-ATPase, including perineurial cells, endothelial cells of blood vessel, and Schwann cells, as well as the nodal axolemma,[50] measurements of nerve Na^+, K^+-ATPase

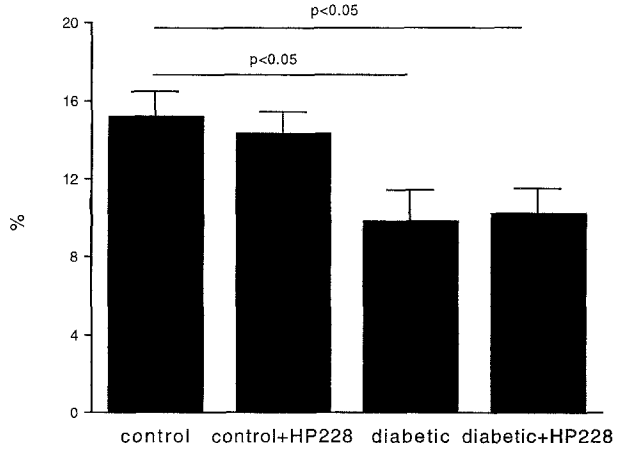

Fig 4. Proportion of axons with diameter greater than 7 μm in the sciatic nerve of control and diabetic rats with or without treatment with HP228. Data are the mean ± SEM; n = 6 to 7.

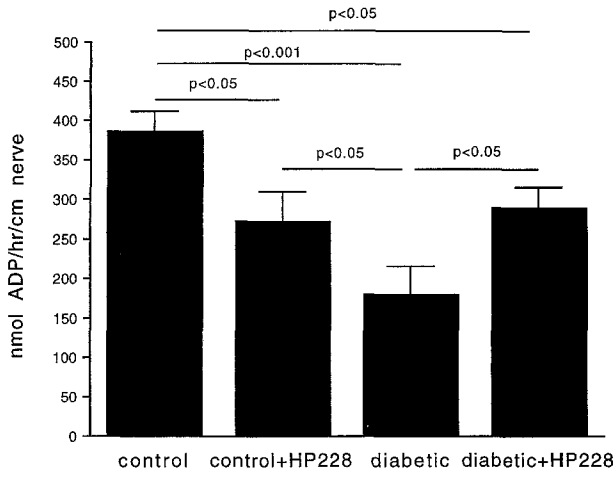

Fig 5. Na^+,K^+-ATPase activity in homogenates of sciatic nerve from control and diabetic rats with or without HP228 treatment. Data are the mean ± SEM; n = 9 to 11.

activity may not directly reflect events specific to the nodal Na^+,K^+-ATPase. While it is clear that HP228 has effects on both homogenate Na^+,K^+-ATPase activity and NCV in diabetic rats, whether the protection of the former underlies the maintenance of the latter remains to be established.

It is notable that, unlike either ARIs or vasoactive agents, HP228 was not effective in preventing the initial decline in MNCV during the first 4 weeks of diabetes but prevented further MNCV slowing over the following 4 weeks. A partial early effect on SNCV was noted. Other agents that have shown a similar pattern of efficacy include gangliosides, which do not prevent short-term conduction slowing[45,51] but do so after a longer duration of diabetes,[52] and the ACTH analog ORG 2766, which was effective against the attenuated maturational increase in NCV of young diabetic rats only after 6 to 8 weeks of treatment.[53] Both gangliosides[54] and ORG 2766[53] prevent the axonal atrophy that develops after weeks of diabetes[13] and which may be expected to contribute to progressive conduction slowing. However, we were unable to show an effect of HP228 on axonal atrophy in diabetic rats, indicating that the effect on NCV was unlikely related to maintenance of axonal caliber.

HP228 shares an amino acid motif at positions 6 to 10 with ACTH and α-MSH. These peptides and a range of related fragments have been reported to possess neurotrophic and neuroprotective properties in cultured cells and also in animal models of peripheral neuropathy.[55] HP228 may therefore act via mechanisms common to those of the melanocortins and their analogs. A number of melanocortin (MC) receptors have been described, of which MC_5 may be located in peripheral nerve.[55] Ligand binding to presumed MC receptors on embryonic dorsal root ganglion cells in vitro enhances cyclic adenosine monophosphate (cAMP) levels, presumably via activation of adenylate cyclase, and may thereby initiate signal transduction cascades.[56] Interestingly, adenylate cyclase activity is impaired in diabetic nerve,[57] and compounds that increase nerve cAMP levels have also been shown to enhance nerve Na^+,K^+-ATPase activity[48] and prevent conduction slowing in diabetic rats.[58-59] Whether HP228 stimulates adenylate cyclase or other signal transduction pathways is not yet known, but it is plausible that it shares signal transduction mechanisms with the melanocortins and other stimulators of adenylate cyclase that protect nerve conduction from the effects of diabetes. These possibilities await further investigation.

This study shows that HP228 attenuates NCV deficits after prolonged, but not acute, periods of diabetes. This may be because of a slow mechanism of action of HP228 or because the continuing decline of NCV in diabetes arises from a variety of pathogenic factors of which only some are arrested by HP228. Further studies involving longer durations of diabetes are clearly warranted to assess the long-term impact of HP228 on nerve structure and function such as those performed using ARIs and other therapeutic agents.[60-62]

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