# Erythropoietin both protects from and reverses experimental diabetic neuropathy

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**Erythropoietin (EPO) possesses generalized neuroprotective and neurotrophic actions. We tested the efficacy of recombinant human EPO (rhEPO) in preventing and reversing nerve dysfunction in streptozotocin (STZ)-induced diabetes in rats. Two days after STZ [60 mg/kg of body weight (b.w.), i.p.], diabetic animals were administered rhEPO (40 $\mu$g/kg of b.w.) three times weekly for 5 weeks either immediately (preventive) before or after a 5-week delay (therapeutic) after induction of hyperglycemia or at a lower dose (8 $\mu$g/kg of b.w. once per week) for 8 weeks (prolonged). Tail-nerve conduction velocities (NCV) was assessed at 5 and 11 weeks for the preventive and therapeutic schedule, respectively. Compared to nondiabetic rats, NCV was 20% lower after 5 weeks in the STZ group, and this decrease was attenuated 50% by rhEPO. Furthermore, the reduction of $Na^+$,$K^+$-ATPase activity of diabetic nerves (by 55%) was limited to 24% in the rhEPO-treated group. In the therapeutic schedule, NCV was reduced by 50% after 11 weeks but by only 23% in the rhEPO-treated group. rhEPO treatment attenuated the decrease in compound muscle action potential in diabetic rats. In addition, rhEPO treatment was associated with a preservation of footpad cutaneous innervation, as assessed by protein gene product 9.5 immunostaining. Diabetic rats developed alterations in mechanical and thermal nociception, which were partially reversed by rhEPO given either in a preventative or therapeutic manner. These observations suggest that administration of rhEPO or its analogues may be useful in the treatment of diabetic neuropathy.**

Polyneuropathy is the most common complication of diabetes mellitus, occurring in >50% of patients who have been hyperglycemic for >15 years (1, 2). Neuropathy contributes the greatest morbidity and mortality and severely impairs the quality of life (3, 4) because of paresthesia, pain, and neuropathic injury, the leading cause of nontraumatic amputation in the U.S. Hyperglycemia is critical for the development and progression of diabetic neuropathy (1, 2), with the two main pathogenic hypotheses focusing on a metabolic vs. vascular etiology. Despite many studies of human and experimental diabetic neuropathy, the current therapeutic arsenal is very poor.

We have previously shown that recombinant human erythropoietin (rhEPO) crosses the blood–brain barrier and has a protective effect in animal models of cerebral ischemia and traumatic injury (5). In primary neuronal cultures or neuronal cell lines and in cerebral ischemia, rhEPO protects from apoptosis (6, 7). rhEPO also reduces injury in experimental autoimmune encephalomyelitis, injury of spinal cord, or sciatic nerve compression (8). i.v. rhEPO is well tolerated and beneficial in patients with acute ischemic stroke (9). Prior work has documented that, within the sciatic nerve, both neurons and Schwann cells express the EPO receptor, which is up-regulated after injury (10). Furthermore, a potential beneficial role for rhEPO in mechanical peripheral nerve injury has been recently shown, for which rhEPO treatment protects dorsal root ganglion neurons from undergoing apoptosis (11).

The present study is aimed at investigating the efficacy of rhEPO in preventing and/or treating peripheral diabetic neuropathy. To accomplish this, we studied rats with streptozotocin (STZ)-induced diabetes, which, like human diabetic neuropathy, have alterations in nociceptive thresholds (thermal and mechanical). In this model, we evaluated thermal and mechanical nociceptive thresholds, supplemented by nerve conduction velocity (NCV) and compound muscle action potentials (CMAP), and sciatic nerve $Na^+$,$K^+$-ATPase content.

Some authorities have considered the first fibers affected in diabetic neuropathy to be the small sensory ones (12–14). In addition, recent data show that the degeneration of intraepidermal nerve fiber (IENF), somatic unmyelinated axons, correlates with both the presence and severity of sensory neuropathy (15–18). We have assessed the involvement of IENF in the STZ-diabetic rat model by quantifying density in rat footpad skin by use of protein gene product 9.5 (PGP 9.5), a specific marker. Administration of rhEPO in all these experiments reduces loss of function in the diabetic state.

## Methodology

**Animal Experimentation.** Procedures involving animals and their care were conducted in conformity with the institutional guidelines in compliance with national (Law by Decree No. 116, February 18, 1992, *Gazzetta Ufficiale della Repubblica Italiana*, Suppl. 40) and international laws and policies (European Economic Community Council Directive 86/609, December 12, 1987, in *Official Journal of Law*, p. 358; Guide for the Care and Use of Laboratory Animals, U.S. National Research Council, 1996). The protocols for the proposed investigation were reviewed and approved by the Animal Care and Use Committees of the Istituto di Ricerche Farmacologiche "Mario Negri" (Milan) and the Faculty of Medicine, University of Mersin. After an overnight fast, male Sprague–Dawley (Charles River Breeding Laboratories) or Wistar rats (Selcuk University Medical Faculty, Konya, Turkey) (200–230 and 265–280 g, respectively) received a single injection [60 mg/kg of body weight (b.w.), i.p., in sodium citrate buffer, pH 4.5] of STZ (Sigma–Aldrich). Only STZ-treated rats with urine glucose levels of >15 mM 2 days after STZ injection were included in the study. Rats were randomized and housed two to three per cage with free access to food and water in a 12-hour light/dark cycle. Control animals were age-matched and given saline instead of STZ.

**Experimental Design.** For preventive studies, Sprague–Dawley diabetic rats were treated with rhEPO (Dragon Pharmaceuticals,

Abbreviations: STZ, streptozotocin; EPO, erythropoietin; rhEPO, recombinant human EPO; CMAP, compound muscle action potential; IENF, intraepidermal nerve fiber; NCV, nerve conduction velocity; PGP 9.5, protein gene product 9.5; b.w., body weight.

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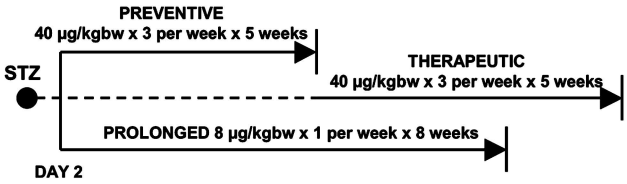


**Fig. 1.** Schematic representation of the treatment schedules used.

Vancouver) 40 $\mu$g/kg of b.w., i.p., three times per week for 5 weeks starting on day 2 ("preventive" schedule), whereas Wistar rats were treated with 8 $\mu$g/kg of b.w., i.p., rhEPO weekly for 4 and 8 weeks ("prolonged" schedule). In the therapeutic schedule, treatment with rhEPO was started 5 weeks after diabetes induction and lasted an additional 5 weeks. Rats were treated with rhEPO (40 $\mu$g/kg of b.w. i.p.) three times per week. The treatment schedules are outlined in Fig. 1. Growth rate, water and food intake, and mechanical and thermal nociceptive thresholds were measured weekly. When indicated, NCV was measured. Plasma glucose, hemoglobin concentration, and hematocrit were evaluated at the end of the experiments. $Na^+,K^+$-ATPase activity in the sciatic nerve was assessed as described (19). An estimate of IENF density was determined by counting nerve fibers visualized by PGP 9.5 immunohistochemistry of the footpad skin by a method previously described (20). Briefly, hind-paw footpad skin was fixed in 2% paraformaldehyde-lysine-periodate for 24 h at 4°C, cryoprotected overnight, and serially cut with a cryostat to obtain 20-$\mu$m sections. Three sections were randomly selected and immunostained with polyclonal anti-PGP 9.5 (Biogenesis, Poole, U.K.) by using a free-floating protocol described by McCarthy *et al.* (21). Three blinded observers counted the total number of IENF in each section under a light microscope at high magnification, with the assistance of a microscope-mounted video camera. Individual fibers were counted as they crossed the dermal–epidermal junction, whereas secondary branching within the epidermis was excluded from the quantification. The length of the epidermis was measured by using a computerized system (Microscience, Seattle) and the linear density of IENF obtained.

**Nociceptive Thresholds.** Thermal nociceptive threshold to radiant heat was quantified by using the paw withdrawal in a hot plate test (22). Withdrawal latency was defined as time between placement on the hot plate and time of withdrawal and licking of hind paw. Each animal was tested twice, separated by a 30-min rest interval. The mechanical nociceptive threshold was quantified by using the Randal–Selitto paw withdrawal test (23) with an analgesy meter (Ugo Basile, Comerio, Italy), which generates a linearly increasing mechanical force. The results represent the maximal pressure (expressed in grams) tolerated by the animals. The thermal nociceptive threshold response utilizes a polysynaptic pathway involving higher centers, whereas the mechanical nociceptive threshold is a monosynaptic response. In agreement with others, in these experiments, diabetes was associated with an increase in the threshold of thermal withdrawal and a decrease in threshold for mechanical stimulation. Rats were accustomed to the devices 3 days before performing the tests. At each time point, animals were tested with three trials, and the values were averaged.

**Electrophysiological Techniques.** Antidromic tail-nerve conduction velocity was assessed by using a Myto EBNeuro electromiograph (EBNeuro, Firenze, Italy), as described (24). The latency of potential recorded in the two sites after nerve stimulation (stimulus duration, 100 msec; filter, 1 Hz to −5 MHz) was determined (peak to peak), and NCV was calculated accordingly. Standardized electromyography and nerve conduction techniques were used for recording CMAP of the gastrocnemius muscle (25). Data were collected with a MP100 acquisition system (Biopac Systems, Santa Barbara, CA).

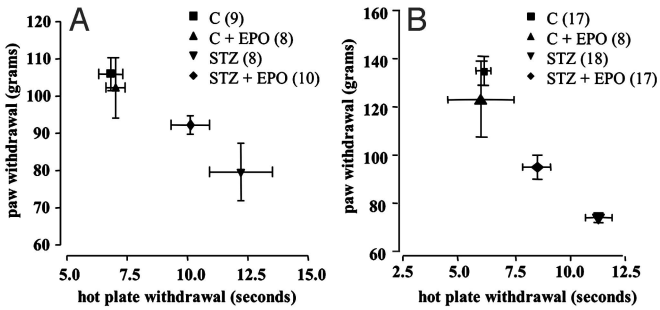


**Fig. 2.** rhEPO prevents and restores changes in thermal and mechanical thresholds in diabetic rats. Control or STZ-diabetic rats were treated with rhEPO according to the preventive (*A*) or therapeutic (*B*) schedule. Thermal sensitivity threshold (*x* axis) is expressed as withdrawal latency in seconds. Mechanical threshold (*y* axis) is expressed as paw withdrawal latency in grams. Measurements were carried out at 5 (*A*) or 9 (*B*) weeks. Data are the mean ± SEM (number of rats in each group is indicated in parentheses).

## Results

After 5, 8, or 11 weeks, all diabetic rats remained hyperglycemic, and rhEPO treatment did not significantly affect blood glucose levels (not shown). Hematocrit in rhEPO-treated rats, either control or diabetic, was significantly increased after week 5 for both the preventive and therapeutic schedule of treatments in diabetic animals (45% and 28% above-normal animals, respectively). After 8 weeks for the prolonged preventive schedule, a rise of 40% above normal was observed.

**Effects of Diabetes and rhEPO Treatment on Mechanical and Thermal Nociceptive Thresholds.** Fig. 2*A* shows the hind-paw thermal- and force-withdrawal (mechanical) thresholds, measured 5 weeks after induction of diabetes, in the experiments when rhEPO was given with the short preventive schedule. After STZ treatment, the thermal response latency and force withdrawal thresholds significantly changed from week 2 (data not shown) until week 5 (Fig. 2*A*). rhEPO administration did prevent (STZ+EPO vs. STZ $P < 0.05$ by Tukey–Kramer test) the increases in thermal nociceptor latency and the decrease in mechanical thresholds in diabetic rats (Fig. 2*A*). rhEPO treatment did not change the thermal response latencies in non-STZ-treated rats at any time point.

In Fig. 2*B*, the hind-paw thermal- and force-withdrawal thresholds in the therapeutic schedule experiment at week 9 are presented. As in the preventive study, diabetic rats showed thermal hypoalgesia and also a decrease in the mechanical thresholds at 5 weeks when groups were randomized to treatments in the therapeutic experiment. In this therapeutic modality, rhEPO was able to significantly ameliorate the thermal response latency from week 7 until week 11. The force-withdrawal threshold in diabetic rats was significantly lower (by 30–46%) at all time points than for control rats. rhEPO treatment partially restored the diabetic mechanical hyperalgesia.

**Effects of Diabetes and EPO Treatment on Electrophysiological Parameters.** The results on tail NCV measured 5 weeks after STZ injection showed that the observed reduction in NCV in diabetic group (−21%) was prevented by ≈50% by rhEPO (control, 32.7 ± 0.52; STZ, 25.7 ± 0.46; STZ plus EPO, 28.1 ± 0.44; m/sec, data are the mean ± SEM). When rhEPO was administered according to the therapeutic schedule, the NCV reduc-

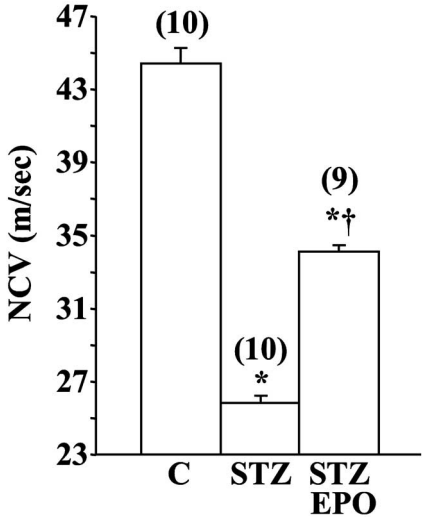


**Fig. 3.** rhEPO restores the decrease in NCV in diabetic rats. Experimental design was the same as in Fig. 1. Tail NCV was measured at 11 weeks (therapeutic schedule). Data are expressed as m/sec and are the mean ± SEM (number of rats is indicated in parentheses). *, $P < 0.001$ vs. nondiabetic control by Tukey–Kramer test; †, $P < 0.01$ vs. STZ.

tion in diabetic group at week 11 was 42% as compared with nondiabetic controls, and rhEPO partially counteracted this decrease (Fig. 3). We also evaluated the CMAP in the gastrocnemius muscle. As shown in Fig. 4, the peak-to-peak amplitude of sciatic nerve CMAP, which mainly reflects axonal dysfunction, was decreased by 30% in diabetes, an effect significantly attenuated by rhEPO, administered in the prolonged preventive schedule.

**Footpad Intraepidermal Nerve Fiber Density.** In diabetic rats, the IENF density is unchanged at 5 weeks of diabetes (data not shown), but it was significantly reduced in diabetic rats at week 11 (end of therapeutic schedule). Fig. 5 shows a representative microphotograph of PGP 9.5 immunostaining of IENF in the footpad of control (Fig. 5*A*), diabetic (Fig. 5*B*) and rhEPO-treated diabetic rats (Fig. 5*C*). It is clearly seen that diabetic rats have a marked decrease in cutaneous innervation density; this is completely reversed by rhEPO administration. A quantification of IENF density, indicating a statistically significant protective effect of rhEPO, is shown in Fig. 5*D*.

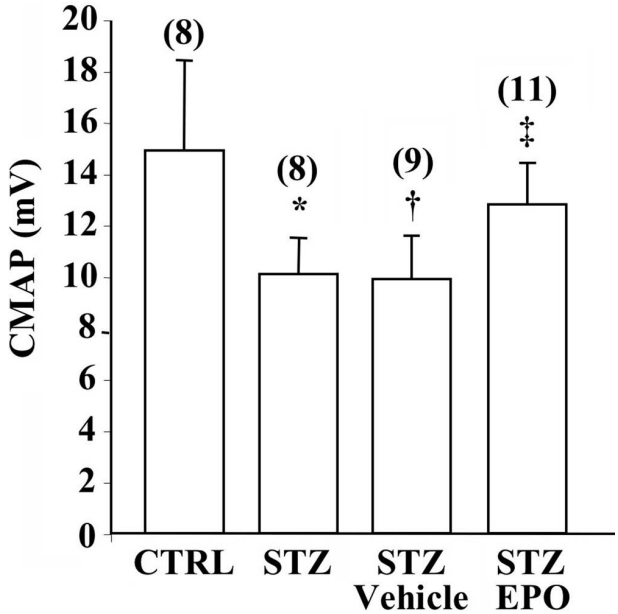


**Fig. 4.** rhEPO prevents the decrease in CMAP in diabetic rats. CMAP was evaluated at week 8. rhEPO was administered according to the prolonged preventive schedule. Data are expressed as mV and are the mean ± SEM (number of rats is indicated in parentheses). *, $P < 0.005$ vs. nondiabetic control by Tukey–Kramer test; †, $P < 0.005$ vs. nondiabetic control; ‡, $P < 0.01$ vs. diabetic groups.

**Effects of Diabetes and rhEPO Treatment on $Na^+,K^+$-ATPase Activity.** As expected, $Na^+,K^+$-ATPase activity was significantly reduced in sciatic nerve from diabetic rats. At 5 weeks (preventive schedule), $Na^+,K^+$-ATPase activity was reduced (−55%) by diabetes, and this reduction was prevented by rhEPO (control, 1.44 ± 0.23; control plus EPO, 1.48 ± 0.14; STZ, 0.65 ± 0.27; STZ + EPO, 1.09 ± 0.08; μmol/min per mg protein; data are the mean ± SEM). When rhEPO was administered according to the therapeutic schedule, the reduced $Na^+,K^+$-ATPase activity (−50%) was also restored (Fig. 6).

## Discussion

The results of this study demonstrate an amelioration effect of systemically administered rhEPO in a rat model of peripheral diabetic neuropathy. In this model, rhEPO partially reversed diabetes-induced loss in nerve functions (NCV and CMAP), cutaneous innervation (IENF density), $Na^+,K^+$-ATPase activity, and impairment in nociceptive thresholds. rhEPO was effective with a preventive schedule where it was given immediately, after the induction of diabetes when the destruction of the β cells is already permanent but before the onset of neuropathy, and also in a therapeutic schedule, i.e., administered when neuropathy was evident.

EPO could modulate on several of the pathogenic pathways implicated in peripheral diabetic neuropathy. This complication is thought to arise from biochemical changes (e.g., protein glycation of cellular proteins, exaggerated flux through the polyol pathway, reduced $Na^+,K^+$-ATPase, and neurotrophic factors. In addition, increased oxidative stress (26) and vascular alterations [decreased microvascular blood flow, increased vascular resistance, and altered vascular permeability (27, 28)] are believed to lead to functional (decreased nerve conduction velocity and nerve blood flow) and structural abnormalities (29, 30). In particular, in STZ-diabetic rats, there are evidences of apoptosis of dorsal root ganglia sensory neurons and Schwann cells (31), possibly due to oxidative stress driven by hyperglycemia (32, 33). Our prior work has shown that EPO prevents neuronal apoptosis *in vitro* in hypoxic neurons and *in vivo* in cerebral ischemia in rats (7). Further, a recent study has found that rhEPO prevents apoptosis in the dorsal root ganglia in rats subjected to a mechanical injury of the sciatic nerve (11). The neurotrophic action of rhEPO (7) could also be important for the effects reported in this paper.

Another pathogenic component of peripheral diabetic neuropathy is inflammation, and we previously reported that experimental diabetic neuropathy is associated with endoneurial induction of inflammatory cytokines and macrophage infiltration (34, 35). This could also be affected by rhEPO, because rhEPO decreases brain inflammation in models of cerebral ischemia (36) and experimental autoimmune encephalomyelitis (37).

Microvascular changes in diabetes mellitus are a final common pathway for late complications, including nerve, heart, kidney, and eye. Studies in humans and in experimental diabetes have shown a reduced nerve blood flow and endoneurial hypoxia in the peripheral nerves (29), and rhEPO was reported to ameliorate neurovascular dysfunction in models of subarachnoid hemorrhage and spinal cord injury (38, 39). In the neurovascular

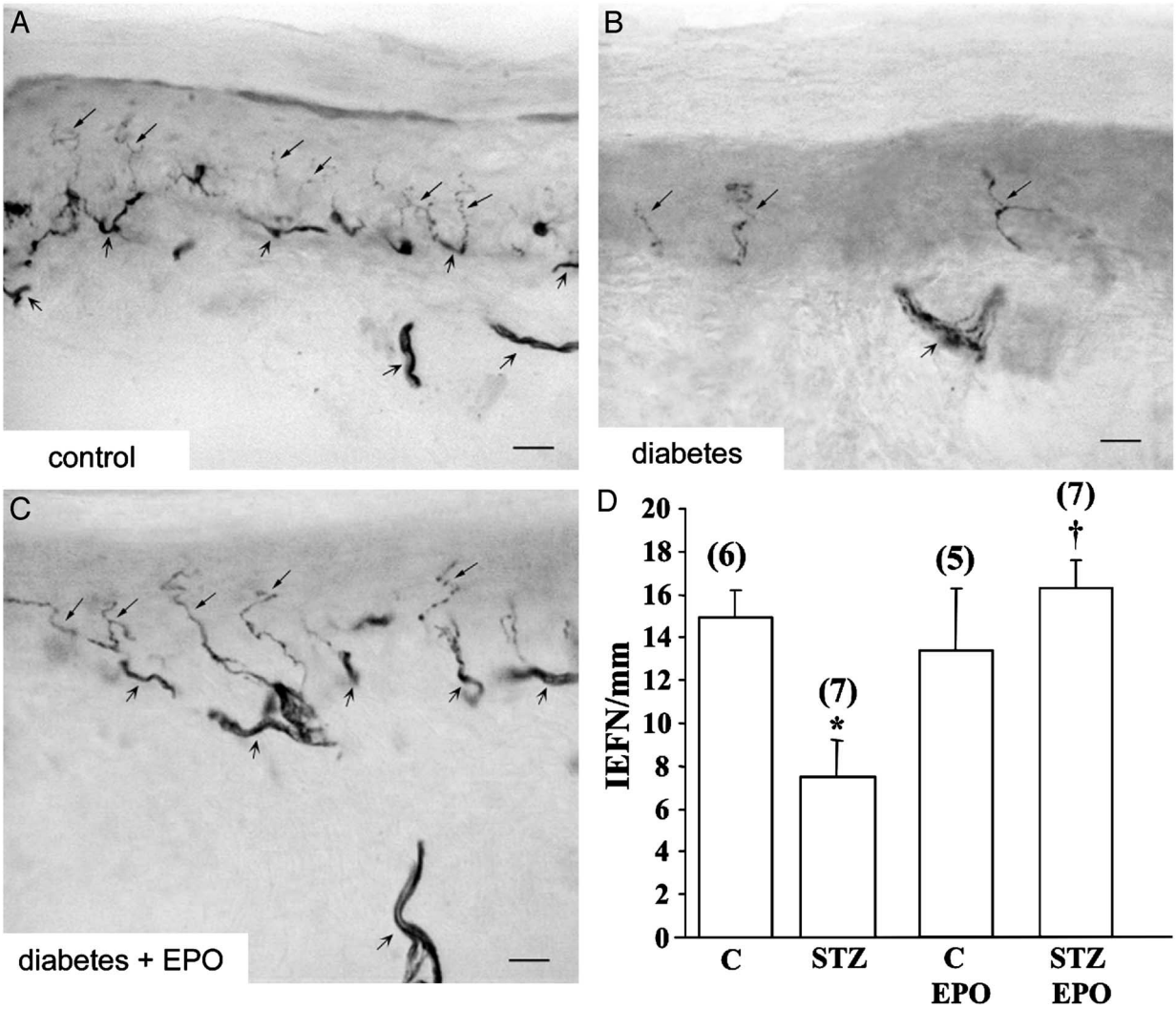


**Fig. 5.** rhEPO restores the loss of intraepidermal fibers in diabetic rats. Hind-paw skin biopsy in control (*A*), STZ-diabetic at 5 weeks (*B*), and after 5 weeks of rhEPO treatment initiated after 5 weeks of diabetes (*C*). Microphotographs are PGP 9.5 immunostaining in 20-$\mu$m-thick sections. (Bar = 30 $\mu$m.) Small arrows indicate IENF, and large arrows indicate dermal nerve bundles. Note the fragmented and discontinuous PGP 9.5 immunoreactivity in IENF indicative of axonal degeneration. (*D*) Quantification of IENF density. Data are expressed as the number of linear density of IENF and are the mean ± SEM (number of rats is indicated in parentheses). *, $P < 0.05$ vs. nondiabetic control by Tukey–Kramer test; †, $P < 0.05$ vs. untreated diabetic rats.

context, angiogenesis helps perfusion in metabolically compromised tissue, and angiogenesis after rhEPO has been reported *in vitro* and *in vivo* (40).

The beneficial efficacy of EPO on NCV and nociception was associated with an effect on IENF density, evaluated by immunohistochemistry by using an anti-PGP 9.5 antibody. PGP 9.5 is a cytoplasmic ubiquitin C-terminal hydrolase expressed in all types of efferent and afferent peripheral nerve fibers. Skin biopsies obtained from patients with diabetes mellitus show uniform reduction in the content of PGP 9.5 (41, 42). Quantification of epidermal axon number in skin biopsies confirmed the loss of cutaneous nerve fibers in diabetic subjects with symptoms of neuropathy, and this reduction correlates with electrophysiological and somatosensory deficits (18, 43, 44). Consistent with these observations, we observed a severe reduction in cutaneous innervation of footpad skin of STZ-diabetic rats. Our results are in agreement with recent findings in genetically diabetic C57BL/Ks J-M+/+Lepr$^{db}$ (db/db) mice and STZ-diabetic mice in which cutaneous innervation is significantly reduced (17, 45). The protective effect of rhEPO on the decrease in IENF density could explain the observed protection from impairment of thermal and, to a lesser extent, mechanical sensitivities. Moreover, the finding that rhEPO prevents the decrease in CMAP in diabetic rats suggests that rhEPO also prevents the loss of functional nerve fibers in the gastrocnemus or improves their functionality.

Some neurotrophic factors (like NGF) prevent thermal hypoalgesia by beneficial effects on small-diameter fibers, whereas others (like neurotrophin-3) selectively protect large sensory neurons (46–49). The development of thermal hypoalgesia in STZ-diabetic rats was partially prevented and restored by rhEPO, which had no effect in nondiabetic animals. Thus, beneficial rhEPO effects appear to be selectively directed to abnormal nociception in diabetes without effects on normal thermal nociception. These results are in agreement with action of some neurotrophic factors but differ from others, like NGF, which, while attenuating thermal hypoalgesia in diabetic rats, also reduced nociceptive threshold in controls (50, 51).

Despite the complexity of nociceptive responses in diabetic neuropathy (52), our data confirm previous results showing that STZ diabetes in rats is associated with mechanical hyperalgesia (53, 54). It seems that, in contrast to thermal nociception,

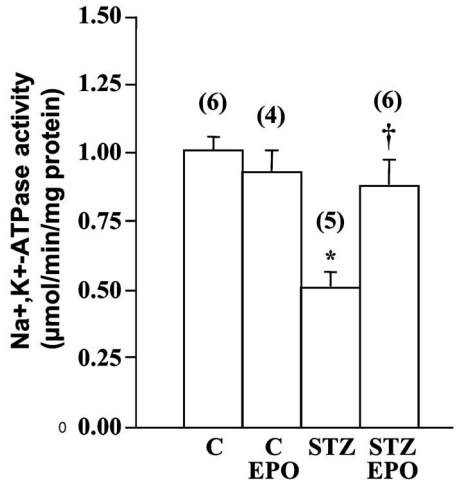


**Fig. 6.** rhEPO restores the decrease in $Na^+,K^+$-ATPase activity in diabetic rats. Experimental design was the same as in Fig. 1. $Na^+,K^+$-ATPase activity was measured at 11 weeks (therapeutic schedule). Data are expressed as $\mu$mol/min per mg protein and are the mean ± SEM (number of rats is indicated in parentheses). *, $P < 0.001$ vs. nondiabetic control by Tukey–Kramer test; †, $P < 0.001$ vs. STZ.

substance P is not involved in mechanical hyperalgesia, where GABA and opiates seem to play a major role (54). These differences in the neuromediators involved, as well as spinal vs. supraspinal sensory processing, might explain the differential effect of rhEPO on the different nociceptive thresholds.

At the biochemical level, potential etiologic mechanisms to explain the slowing of NCV during hyperglycemia include decreased activity of $Na^+,K^+$-ATPase in peripheral nerves and atrophy of large myelinated fibers (30). We previously observed that the $Na^+,K^+$-ATPase in the peripheral nerves is sensitive to environmental hypoxic conditions (55). It is important to note that our previous work has shown that, in diabetic patients, both the total number of fibers and the specific activity of $Na^+,K^+$-ATPase are decreased (56), indicating that diabetic neuropathy is not only due to a loss in nerve fibers but also to a decrease in their functionality. We report here that rhEPO prevents and restores the loss of $Na^+,K^+$-ATPase activity in diabetic rats, suggesting that rhEPO might also ameliorate the functionality of existing fibers, in agreement with the neurotrophic effect of rhEPO reported in different experimental models (7, 57).

A number of agents have been previously shown to have activity preventing the onset of diabetic neuropathy (29). A few have been shown to have some reversing activity. *N*-acetylcysteine, a hydrophilic antioxidant and sulfydryl donor, has been shown to reverse NCV and nerve blood flow after 1 month of untreated diabetes and improved sciatic nerve mylenated fiber regeneration (58). A neurotrophic peptide of prosaposin proved efficacious in preventing onset of large and small fiber dysfunction and thermal hypoalgesia in diabetic rats (50). Tumor necrosis factor $\alpha$ (TNF-$\alpha$) acutely induces hyperalgesia, and prosaposin-derived peptide counteracts this activity (59). By additionally reducing TNF production, prevention and reversal, along with almost complete normalization of sciatic nerve myelinated fibers, has been reported (60). In one successful experiment using a probucol derivative, coadministration of a NO synthase inhibitor abolishes the effects of antioxidants, suggesting that the benefits arise from effects on the neurovasculum (61). Finally, lipoic acid has been shown in diabetic patients to improve function and reduce symptoms in patients with established diabetic polyneuropathy (62).

These observations strongly suggest that administration of rhEPO may be useful in the treatment of diabetic neuropathy. However, although in acute brain injury such as cerebral ischemia or brain trauma, a single injection of rhEPO was sufficient to obtain a protective effect (5), in the present study, rhEPO was administered over a long period (5–8 weeks). With this schedule, a marked increase in the hematocrit was observed. This could represent a potentially serious side effect and increase the risk of cerebrovascular accidents. Therefore, in the setting of chronic diseases, it will be important to develop nonerythropoietic analogues of EPO. Significant progress has been made toward this goal: we recently have reported on the efficacy of asialoerythropoietin, an analogue with a markedly reduced half life (8). Although experiments have shown that asialoEPO protects from sciatic nerve compression, further testing will be required to determine whether asialoEPO is also active in diabetic neuropathy.

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