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PROGESTERONE AND ITS DERIVATIVES ARE NEUROPROTECTIVE AGENTS IN EXPERIMENTAL DIABETIC NEUROPATHY: A MULTIMODAL ANALYSIS

E. LEONELLI,[a1] R. BIANCHI,[b1] G. CAVALETTI,[c] D. CARUSO,[d] D. CRIPPA,[e] L. M. GARCIA-SEGURA,[f] G. LAURIA,[g] V. MAGNAGHI,[a] I. ROGLIO[a] AND R. C. MELCANGI[a]*

[a]*Department of Endocrinology and Center of Excellence on Neurodegenerative Diseases, University of Milan, Via Balzaretti 9, 20133 Milano, Italy*

[b]*Department of Molecular Biochemistry and Pharmacology, "Mario Negri" Institute for Pharmacological Research, Milano, Italy*

[c]*Department of Neurosciences and Biomedical Technologies, University of Milan "Bicocca," Monza, Italy*

[d]*Department of Pharmacological Sciences, University of Milan, Milano, Italy*

[e]*Schering SpA, Segrate, Milano, Italy*

[f]*Instituto Cajal, C.S.I.C., Madrid, Spain*

[g]*Neuromuscular Diseases Unit, National Neurological Institute "Carlo Besta," Milano, Italy*

Abstract—One important complication of diabetes is damage to the peripheral nervous system. However, in spite of the number of studies on human and experimental diabetic neuropathy, the current therapeutic arsenal is meagre. Consequently, the search for substances to protect the nervous system from the degenerative effects of diabetes has high priority in biomedical research. Neuroactive steroids might be interesting since they have been recently identified as promising neuroprotective agents in several models of neurodegeneration. We have assessed whether chronic treatment with progesterone (P), dihydroprogesterone (DHP) or tetrahydroprogesterone (THP) had neuroprotective effects against streptozotocin (STZ)-induced diabetic neuropathy at the neurophysiological, functional, biochemical and neuropathological levels. Using gas chromatography coupled to mass-spectrometry, we found that three months of diabetes markedly lowered P plasma levels in male rats, and chronic treatment with P restored them, with protective effects on peripheral nerves. In the model of STZ-induced of diabetic neuropathy, chronic treatment for 1 month with P, or with its derivatives, DHP and THP, counteracted the impairment of nerve conduction velocity (NCV) and thermal threshold, restored skin innervation density, and improved Na^+,K^+-ATPase activity and mRNA levels of myelin proteins, such as glycoprotein zero and peripheral myelin protein 22, suggesting that these neuroactive steroids, might be useful protective agents in diabetic neuropathy. Interestingly, different receptors seem to be involved in these effects. Thus, while the expression of myelin proteins and Na^+,K^+-ATPase activity are only stimulated by P and DHP (i.e. two neuroactive steroids interacting with P receptor, PR), NCV, thermal nociceptive threshold and intra-epidermal nerve fiber (IENF) density are also affected by THP, which interacts with GABA-A receptor. Because, a therapeutic approach with specific synthetic receptor ligands could avoid the typical side effects of steroids, future experiments will be devoted to evaluating the role of PR and GABA-A receptor in these protective effects.

Key words: diabetes, neuroactive steroids, myelin, rat, sciatic nerve, streptozotocin.

Human diabetic neuropathy (HDN) occurs in more than 50% of diabetic patients and involves a spectrum of functional and structural changes in peripheral nerves. Early disorders of nerve function include slowing in nerve conduction velocity (NCV) followed by axonal degeneration, paranodal demyelination and loss of myelinated fibers (Sugimoto et al., 2000; Vinik et al., 2000). Experimental diabetic neuropathy shares a number of features with HDN, such as the structural, functional and biochemical alterations (Dyck et al., 1988; Cameron and Cotter, 1994; Yagihashi, 1997; Biessels et al., 1999; Eckersley, 2002). The reduction of Na^+,K^+-ATPase activity, together with the decrease in NCV, is the hallmark of diabetic neuropathy (Greene et al., 1989; Berry, 1997). HDN severely impairs the quality of life because of persistent sensory symptoms, including pain and abnormal thermal perception. Alterations in nociceptive threshold can be reproduced in rats with streptozotocin (STZ)-induced diabetes and can be partially prevented and restored by erythropoietin and prosaposin-derived peptide treatments (Calcutt et al., 2000; Bianchi et al., 2004).

At the pathological level, the decrease of intra-epidermal nerve fiber (IENF) density in diabetics with symptoms of neuropathy correlates with electrophysiological and psychophysical abnormalities (Periquet et al., 1999; Lauria et al., 2003, 2005). Likewise, IENF density is significantly reduced in *db*/*db* and STZ-diabetic rats and mice (Underwood et al., 2001; Christianson et al., 2003; Bianchi et al., 2004).

Current treatment of diabetic neuropathy relies on the control of glycemic, oxidative stress and neural and vascular risk factors (Vincent et al., 2004; Tesfaye et al.,

[1] E.L. and R.B. contributed equally to this study.

*Corresponding author. Tel: +39-02-50318238; fax: +39-02-50318204.
E-mail address: roberto.melcangi@unimi.it (R. C. Melcangi).

Abbreviations: bp, base pair; c.p.m., counts per minute; DHP, dihydroprogesterone; GC, gas chromatography; HDN, human diabetic neuropathy; IENF, intra-epidermal nerve fiber; MS, mass spectrometer; NCI, negative chemical ionization; NCV, nerve conduction velocity; P, progesterone; PMP22, peripheral myelin protein 22; PR, progesterone receptor; PREG, pregnenolone; P0, glycoprotein zero; STZ, streptozotocin; THP, tetrahydroprogesterone; 3alpha-HSD, 3alpha-hydroxysteroid dehydrogenase.

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2005), but this does not fully prevent its occurrence or progression. Recent findings suggest that neuroactive steroids might exert neuroprotective effects and may be promising in therapy. Our and other laboratories have found that neuroactive steroids themselves and synthetic ligands that interact with their receptors can counteract peripheral nerve degeneration occurring in experimental physical trauma, aging, or in hereditary demyelinating diseases (Koenig et al., 1995; Melcangi et al., 2000, 2003; Azcoitia et al., 2003; Sereda et al., 2003). Similar effects were observed with molecules that induce synthesis *in situ* of neuroactive steroids (e.g. ligands for peripheral benzodiazepine receptors) (Leonelli et al., 2005). We reported that progesterone (P) and its derivatives dihydroprogesterone (DHP) and tetrahydroprogesterone (THP) can stimulate the expression of myelin proteins (i.e. glycoprotein zero, P0, and peripheral myelin protein 22, PMP22) (Melcangi et al., 1998, 1999) both in physiological and pathological conditions and preserve the morphology of myelin sheaths (Azcoitia et al., 2003) and axons (Melcangi et al., 2003).

It has been recently reported that P biosynthesis is up-regulated in the spinal cord of rats with STZ-induced diabetes (Saredi et al., 2005). Furthermore, we have observed that P administration results in a significant reduction in the number of fibers with myelin abnormalities in the sciatic nerve of STZ-treated rats (Veiga et al., 2006). These findings suggest that neuroactive steroids, such as P, may represent potential therapeutic tools for the treatment of diabetic neuropathy. Therefore, we hypothesized that P and its reduced metabolites, DHP and THP, might protect peripheral nerves from degeneration induced by diabetes. Accordingly, our study was designed to assess whether P, DHP and THP had neuroprotective effects on STZ-induced neuropathy at neurophysiological, functional, biochemical and neuropathological levels.

EXPERIMENTAL PROCEDURES

Animals and treatments

Animals. Two-month-old male Sprague–Dawley rats, Crl:CD BR (Charles River, Calco, Italy) were housed in the department animal quarters with controlled temperature and humidity. The light schedule was 14-h light and 10-h dark (lights on at 06:30 h). The animals were handled following the European Union Normative (Council Directive 86/609/EEC), with the approval of our Institutional Animal Use and Care Committees. Special care was taken to minimize animal suffering and to reduce the number of animals used to the minimum required for statistical accuracy.

Table 1. Body weight and glucose levels of control, diabetic and diabetic rats treated following experimental protocol 1 with P, DHP, or THP

Animal	Body weight before STZ injection (g)	Body weight at death (g)	Blood glucose at death (mg/dl)
Control	268.5±2.8	594.2±11.5	95±4
Diabetic	269.0±4.8	310.0±13.2***	556±19***
Diabetic+P	263.3±2.9	322.2±20.0***	517±36***
Diabetic+DHP	262.7±2.9	334.3±16.1***	507±29***
Diabetic+THP	265.3±2.1	327.0±13.1***	572±17***

Mean±S.E.M. (n=8).
*** P<0.001 vs. control.

Table 2. Body weight and glucose levels of control, diabetic and diabetic rats treated following experimental protocol 2 with P, DHP, or THP

Animal	Body weight before STZ injection (g)	Body weight at death (g)	Blood glucose at death (mg/dl)
Control	285.8±3.1	502.8±8.3	93±4
Diabetic	290.4±3.8	335.9±16.1***	538±25***
Diabetic+P	292.6±3.1	328.1±18.1***	560±13***
Diabetic+DHP	287.4±3.5	315.3±18.2***	542±19***
Diabetic+THP	280.5±10.3	304.9±25.7***	564±19***

Mean±S.E.M. (n=8).
*** P<0.001 vs. control.

Induction of diabetes. Diabetes was induced by a single i.v. injection of freshly prepared STZ (65 mg/kg; Sigma, Milano, Italy) in citrate buffer 0.09 M pH 4.8. Control animals were injected with 0.09 M citrate buffer at pH 4.8. Hyperglycemia was confirmed 48 h after STZ injection by measuring tail vein blood glucose levels using a Glucomen tester (Menarini, Naples, Italy). Only animals with mean plasma glucose levels above 300 mg/dl were classified as diabetic. Glycemia was also confirmed before the treatment with steroids (two months after STZ injection, see below) and tested at scheduled death, three months after STZ.

Neuroactive steroids. Two different protocols of steroid administration were assessed. In the first one, animals were treated eight times with steroids and injections were administered every 4 days; in the second one, animals were injected every 2 days (i.e. they received 16 treatments of steroids).

In particular, two months after the STZ injection, rats received eight (experimental protocol 1) or 16 (experimental protocol 2) s.c. injections of 1 mg of P, DHP or THP (Sigma) dissolved in 200 μl of sesame oil (3.3 mg/kg body weight). Control rats were injected with 200 μl of vehicle (sesame oil). Injections were administered every four (experimental protocol 1) or two (experimental protocol 2) days. Twenty-four hours after the last treatment, the rats were killed and sciatic nerves and hindpaw footpad skin were rapidly removed and stored according to the protocol of analysis.

Pregnenolone (PREG), P and THP concentrations in plasma

Steroids in plasma were extracted according to Vallée et al. (2000) with minor modification. Briefly, the internal standard deuterium-labeled 17,21,21,21-d_4 PREG, 15 ng/sample, (D4-PREG; synthesized by Dr. P. Ferraboschi, Department of Medical Chemistry, Biochemistry and Biotechnology, University of Milan, Milano, Italy) and methanol with acetic acid 1% were added to the plasma samples (1.5 ml). Samples were then extracted with C18 cartridge (Discovery® DSC-18 cartridge, Supelco, Milano, Italy) and the steroid fraction was eluted with methanol (5 ml).

The derivatization procedures were as follows: the pentafluorobenzyl derivative was formed by adding a mixture of pyridine: Florox® reagent (1:2, v/v; Fluka, Milano, Italy) to the organic residue and heating for 1 h at 70 °C. After evaporation of the excess derivatizing reagent, trimethylsilyl derivatives were formed by adding BSTFA:TMCS 1% (Supelco) in ethyl acetate, for 20 min, at 70 °C.

PREG, P and THP plasma levels were evaluated by gas chromatography (GC), ThermoTrace GC 2000 Series 8 (ThermoQuest

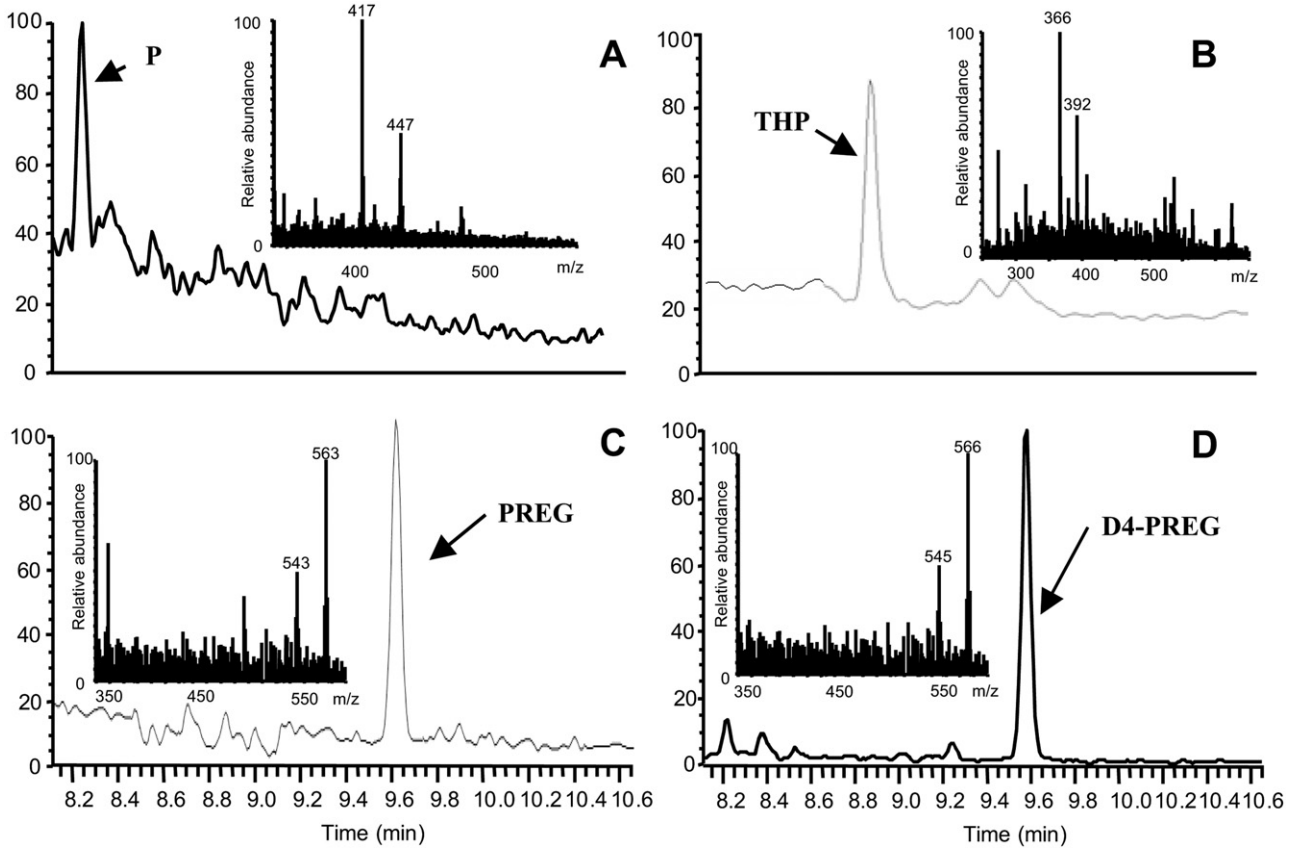

Fig. 1. Representative GC–NCI–MS ion chromatograms of a plasma sample (1.5 ml). The identity of each peak is based on the retention time (RT) and the mass spectrum (see the windows) of pure compounds. (A) Selected ion chromatogram (m/z 417) corresponding to P (RT 8.25 min); (B) selected ion chromatogram (m/z 366) corresponding to THP (RT 8.87 min); (C) selected ion chromatogram (m/z 563) corresponding to PREG (RT 9.60 min); (D) selected ion chromatogram (m/z 566) corresponding to PREG deuterated (D4-PREG, RT 9.57 min).

Finnigan, San Jose, CA, USA) coupled to the mass spectrometer (MS), ThermoQuest Finnigan GC_Q, equipped with an ion trap source operating in negative chemical ionization (NCI) mode with methane as reagent gas. The analyses were done in single ion monitoring (SIM) mode following the ions m/z 563, 543 for PREG, m/z 566, 545 for D4-PREG (as internal standard), m/z 366, 392 for THP and m/z 417, 447 for P. Quantitative analyses were performed on the basis of calibration curves prepared and injected after and before biological samples.

Functional analyses

Thermal nociceptive threshold. The nociceptive threshold to radiant heat was quantified using the hot plate paw withdrawal test as previously described (Bianchi et al., 2004). Briefly, a 40 cm high Plexiglas cylinder was suspended over the hot plate and the temperature was maintained at 50 °C to give a latency of about 10 s for control rats. Paw withdrawal latency was defined as the time between placing the rat on the hot plate and the time of withdrawal, or licking of hind paw, or discomfort manifested by the animal. The test was done every two weeks starting from the second week after STZ injection. Animals were tested twice, with a 30-min interval, in each test section.

NCV. At the end of the treatments antidromic tail NCV was assessed using a Myto EBNeuro electromyograph (EBNeuro, Firenze, Italy) as previously described (Tredici et al., 1998). Briefly, recording ring electrodes were placed distally in the tail. The stimulating ring electrodes were placed 5 cm and 10 cm proximally with respect to the recording point. The latencies of the potentials recorded at the two sites after nerve stimulation were determined (peak-to-peak, stimulus duration 100 ms, filter 1 Hz-5 MHz) and NCV was calculated. All the neurophysiological studies were done under standard conditions in a temperature-controlled room adjacent to the animal housing room.

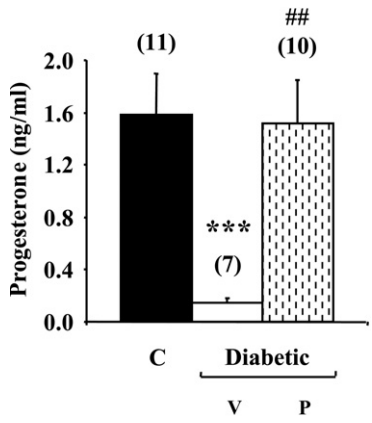

Fig. 2. P level in plasma of control (C) and diabetic rats treated following experimental protocol 2 with vehicle only (V) or with P. Data are expressed as ng/ml and are the mean±S.E.M. (number of rats in each group in parentheses). *** $P<0.001$ vs. control; ## $P<0.01$ vs. diabetic injected with vehicle.

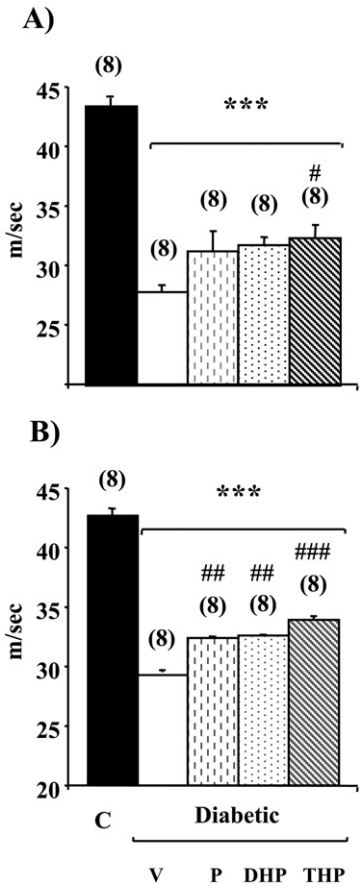

Fig. 3. Following experimental protocol 1, only THP partially restores the decrease in NCV in diabetic rats (A). On the contrary, following experimental protocol 2, not only THP, but also P and DHP, are effective on this parameter (B). Tail NCV was measured at the end of the experiment (12 weeks) in control (C) and diabetic rats treated with vehicle only (V), or with neuroactive steroids. Data are expressed as m/sec and are the mean±S.E.M. (number of rats in each group in parentheses). *** $P<0.001$ vs. control; # $P<0.05$ vs. diabetic injected with vehicle; ## $P<0.01$ vs. diabetic injected with vehicle; ### $P<0.001$ vs. diabetic injected with vehicle.

Skin biopsies. Peripheral nerve damage was assessed by pathological examination on a skin biopsy, quantifying IENF density in the hindpaw footpad (Lauria et al., 2005). Briefly, hindpaws were collected at death. After separating the plantar glabrous skin, which included epidermis and dermis, from the underlying metatarsal bones, 3-mm round samples were taken and immediately fixed in 2% paraformaldehyde-lysine periodate for 24 h at 4 °C, cryoprotected overnight, and serially cut with a cryostat to obtain 20-μm sections. Two sections from each footpad were randomly selected and immunostained with rabbit polyclonal anti-protein gene product 9.5 (PGP 9.5; Biogenesis, Poole, UK) using a free-floating protocol (Lauria et al., 2005). Two observers blinded to the healthy or neuropathic status of rats, independently counted the total number of PGP 9.5-positive 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, and secondary branching within the epidermis was excluded. The length of the epidermis was measured using a computerized system (Microscience Inc., Seattle, WA, USA) and the linear density of IENF (IENF/mm) was obtained.

Biochemical analyses

Na^+,K^+-ATPase activity. Tibial stumps (from the two sciatic nerves) were desheathed at death and homogenized in chilled solution containing 0.25 M sucrose, 1.25 mM EGTA and 10 mM Tris, pH 7.5, at 1:20 (w/v) in a glass-glass Elvejehm-Potter homogenizer (DISA, Milano, Italy), and stored at −80 °C for ATPase determinations. Na^+,K^+-ATPase activity was determined spectrophotometrically as previously described (Bianchi et al., 1988). Protein content in homogenates was determined by Lowry's method (Lowry et al., 1951) with bovine serum albumin as standard.

cRNA probes. The specific pCR®II-TOP0® (Invitrogen, Milano, Italy) plasmids contain, the following inserts: 387 base pair (bp) for P0, 414 bp for PMP22, 290 bp in the case of 18s. The cRNA antisense probes were generated by *in vitro* transcription of different pCR®II-TOP0® specific plasmids in presence of [^{32}P]-CTP (Amersham, Milano, Italy) as labeled nucleotide. All the cRNA probes were obtained with specific activity $>10^8$ counts per minute (c.p.m.)/μg.

RNase protection assay. Total RNA from snap-frozen sciatic nerves was isolated by phenol–chloroform extraction according to the method of Chomczynski and Sacchi (1987). Samples of

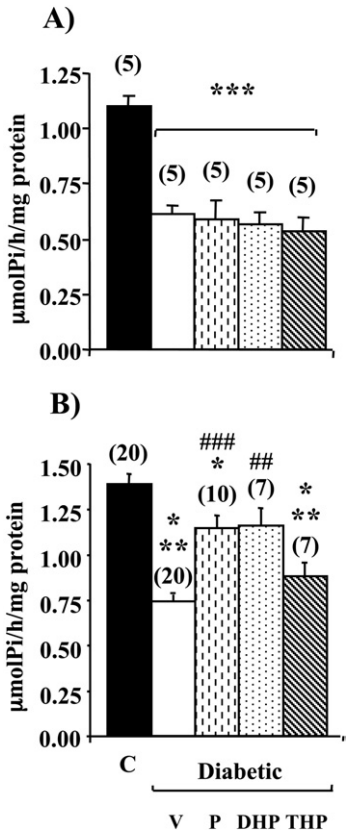

Fig. 4. Following experimental protocol 1, P and its derivatives, DHP and THP, are unable to restore the decrease in tibial Na^+,K^+-ATPase activity in diabetic rats (A). On the contrary, administration of P or DHP, following experimental protocol 2, partially restores the decrease in tibial Na^+,K^+-ATPase activity in diabetic rats. Na^+,K^+-ATPase activity was measured at the end of the experiment (12 weeks). Data are expressed as μmol Pi/h per mg protein and are the mean±S.E.M. (number of rats in each group in parentheses). * $P<0.05$ vs. control; *** $P<0.001$ vs. control; ## $P<0.005$ vs. diabetic injected with vehicle; ### $P<0.001$ vs. diabetic injected with vehicle.

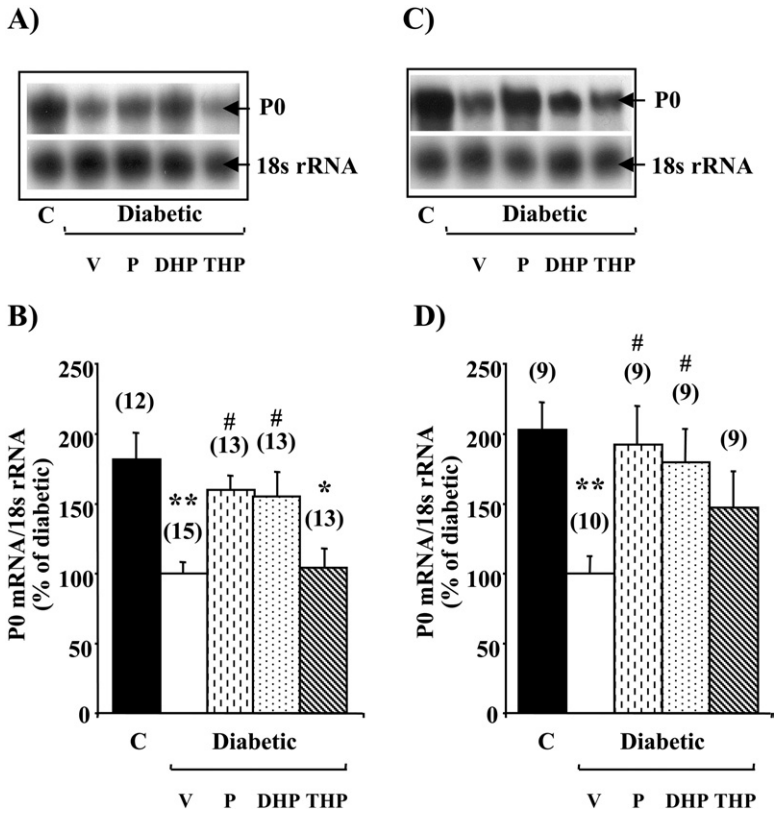

Fig. 5. Following experimental protocol 1 or 2, P and its derivative, DHP, counteract the decrease of P0 mRNA in sciatic nerve of diabetic male rats. (A) Representative RNase protection assay blot of one of the experiments performed with experimental protocol 1. (B) P0 mRNA levels at the end of the experiment (12 weeks) in control (C) and in diabetic rats treated following experimental protocol 1 with vehicle only (V), or with P, DHP, or THP. (C) Representative RNase protection assay blot of one of the experiments performed with experimental protocol 2. (D) P0 mRNA levels at the end of the experiment in C and in diabetic rats treated following experimental protocol 2 with V, or with P, DHP, or THP. The data are percentages of the levels in diabetic rats. The columns represent the means±S.E.M. after normalization with 18s rRNA (number of rats in each group in parentheses). * $P<0.05$ vs. control; ** $P<0.01$ vs. control; # $P<0.05$ vs. diabetic injected with vehicle.

total RNA (10–12 μg) were utilized in the RNase protection assay, as previously described (Magnaghi et al., 2006). Briefly, after ethanol precipitation samples were dissolved in 20 μl of hybridization solution (80% formamide, 40 mM PIPES pH 6.4, 400 mM sodium acetate pH 6.4 and 1 mM EDTA) containing 150,000 c.p.m. of each ^{32}P-labeled cRNA probe and 50,000 c.p.m. of ^{32}P-labeled cRNA 18s probe. After heating at 85 °C for 10 min, the probes were allowed to hybridize the endogenous RNAs at 45 °C overnight. Subsequently, the samples were diluted with 200 μl of RNase digestion buffer (300 mM NaCl, 10 mM Tris–HCl pH 7.4, 5 mM EDTA pH 7.4) containing a 1:400 dilution of an RNase cocktail (1 μg/μl RNase A and 20 U/μl RNase T1) and incubated for 30 min at 30 °C. Then, 10 μg of proteinase K and sodium dodecyl sulfate (10 ml of 20% stock solution) were added to the samples and the mixture was incubated at 37 °C for 15 min. The samples were then extracted with phenol–chloroform and precipitated with ethanol. The pellet was dried and resuspended in loading buffer (80% formamide, 0.1% xylene cyanol, 0.1% Bromophenol Blue, 2 mM EDTA) boiled at 95 °C for 5 min and separated on a 5% polyacrylamide gel, under denaturing conditions (7 M urea). The protected fragments were visualized by autoradiography and their sizes were determined using ^{32}P-end-labeled (T4 polynucleotide kinase) *Msp*I-digested pBR322 fragments.

RNA calculation. The levels of mRNA for P0, PMP22, and 18s rRNA were calculated by measuring the peak densitometric area of the autoradiography analyzed with a Kodak Snap Scanner (Eastman Kodak, Rochester, NY, USA). To ensure that the autoradiographic bands were in the linear range of intensity, different exposure times were used. The mean control value within a single experiment was set to 100 and all the other values were expressed as a percentage of this. Values of controls from different experiments were all within 10%.

Statistical analysis

One-way analysis of variance (ANOVA) followed by the Tukey-Kramer post-test was used.

RESULTS

In our first set of experiments we have tested an experimental protocol of steroid administration demonstrated to be protective in other models of neurodegeneration (Melcangi et al., 1998, 2000; Azcoitia et al., 2003), i.e. animals received eight treatments of 1 mg of steroids and injections were administered every 4 days (experimental protocol 1) and we have compared the effect of this with another one in which we have increased the frequency of treatment, i.e. animals were injected every 2 days and consequently received 16 treatments of steroids (experimental protocol 2).

As shown in Table 1, diabetic rats had high blood glucose at the end of the experiment and, as expected, significantly less weight gain than non-diabetic control rats. Following experimental protocol 1, treatment with P, or its reduced derivatives, DHP and THP, did not significantly modify body weight and blood glucose levels compared with diabetic vehicle-treated rats (Table 1). As shown in Table 2, also with experimental protocol 2, treatment with P, or its reduced derivatives, DHP and THP, did not significantly modify body weight and blood glucose levels compared with diabetic vehicle-treated rats.

Plasma levels of P, PREG (i.e. its precursor), and THP were assessed by GC–NCI–MS (Fig. 1). Diabetes did not affect PREG (data not shown) and THP plasma levels (ng/ml±S.E.M.: 1.09±0.31 in controls vs. 0.89±0.38 in diabetic rats), but three months of diabetes lowered P plasma levels by about 90% compared with the levels in control rats (Fig. 2). Utilizing experimental protocol 2, treatment with P completely restored the normal plasma levels of this neuroactive steroid in diabetic rats.

Diabetes reduced tail nerve NCV by 25% (Fig. 3). Utilizing experimental protocol 1, although either treatment with P and DHP showed a similar tendency, only THP was able to partially, but significantly, restore the reduced NCV (Fig. 3, panel A).

Interestingly with experimental protocol 2, not only THP, but also P and DHP were able to significantly increase NCV (Fig. 3, panel B).

As shown in Fig. 4, diabetic rats showed a 45% reduction in tibial Na^+,K^+-ATPase activity; however, utilizing experimental protocol 1, neither P nor its derivatives were able to significantly modify this parameter (Fig. 4, panel A). On the contrary, utilizing experimental protocol 2 (Fig. 4, panel B), the reduction of this activity occurring in tibial nerve of diabetic rats was significantly restored by P and DHP (by 54% and 57% respectively). THP induced a similar positive pattern (+19%) in Na^+,K^+-ATPase activity, but this did not reach the level of significance.

Diabetes also affected expression of myelin proteins. As shown in Fig. 5, a significant decrease of P0 mRNA

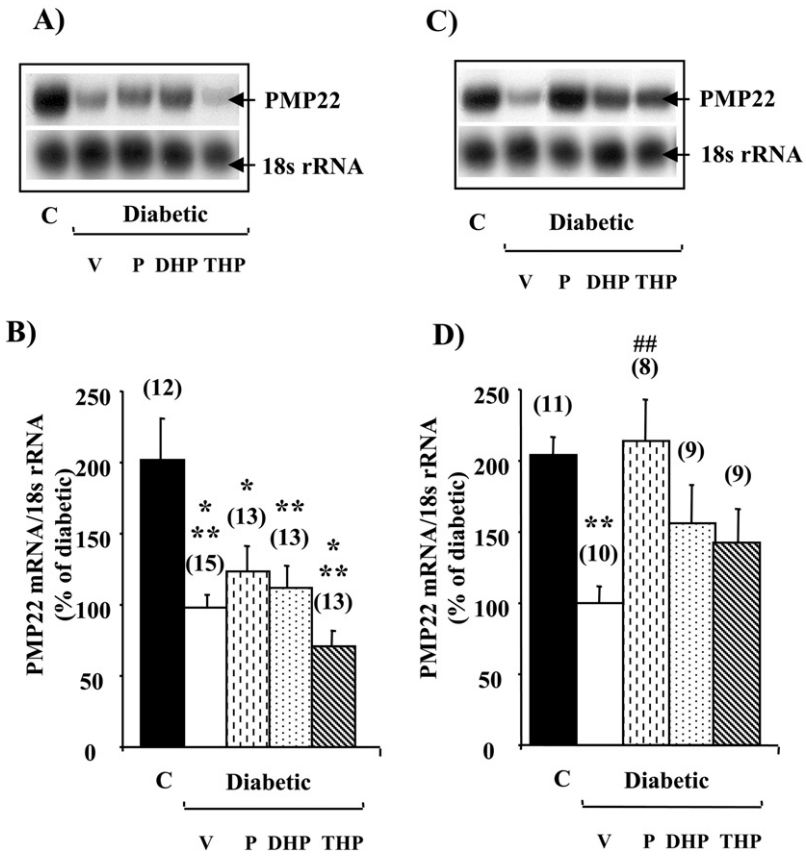

Fig. 6. Following experimental protocol 1, P and its derivatives, DHP and THP, are unable to counteract the decrease of PMP22 mRNA in sciatic nerve of diabetic male rats. On the contrary, P administered following experimental protocol 2 counteracts the decrease of PMP22 mRNA in sciatic nerve of diabetic male rats. (A) Representative RNase protection assay blot of one of the experiments performed with experimental protocol 1. (B) PMP22 mRNA levels at the end of the experiment (12 weeks) in control (C) and in diabetic rats treated following experimental protocol 1 with vehicle only (V), or with P, DHP, or THP. (C) Representative RNase protection assay blot of one of the experiments performed with experimental protocol 2. (D) PMP22 mRNA levels were measured at the end of the experiment in C and in diabetic rats treated following experimental protocol 2 with V, or with P, DHP, or THP. The data are percentages of levels detected in diabetic rats. The columns represent the means±S.E.M. after normalization with 18s rRNA (number of rats in each group in parentheses). * $P<0.05$ vs. control; ** $P<0.01$ vs. control; *** $P<0.001$ vs. control; ## $P<0.01$ vs. diabetic injected with vehicle.

levels occurs in sciatic nerve of STZ-treated male rats. Utilizing experimental protocol 1 (Fig. 5, panels A and B) or 2 (Fig. 5, panels C and D) treatments with P or DHP induced a significant increase of messenger levels of P0, while THP treatment was ineffective.

Similarly, diabetes reduced mRNA levels of PMP22 (Fig. 6), but, in this case, only experimental protocol 2 was effective. Thus, utilizing experimental protocol 1, all the three neuroactive steroids considered were ineffective (Fig. 6, panels A and B), while with experimental protocol 2, the treatment with P achieved a significant increase of PMP22 mRNA (Fig. 6, panels C and D). With this experimental protocol, DHP or THP treatment induced a similar, but not significant tendency.

On the basis of the interesting protective effects observed with the experimental protocol 2, we have extend the analysis on other parameters.

As shown in Fig. 7, diabetes significantly raised thermal threshold by about 60%. Utilizing experimental protocol 2, treatment with P, DHP or THP normalized this parameter in diabetic rats.

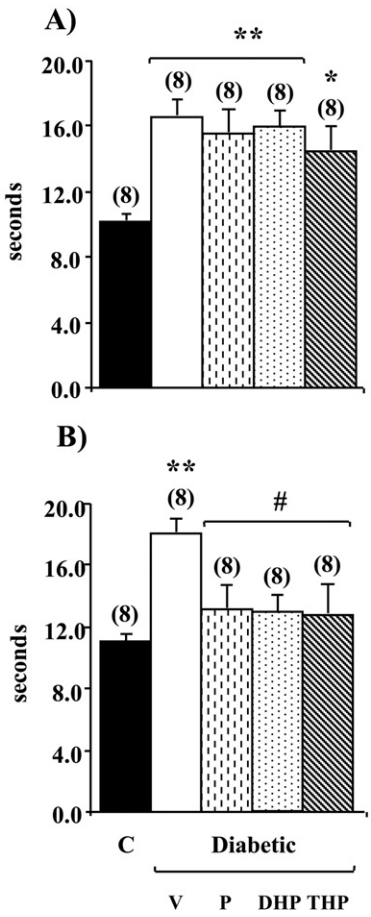

Fig. 7. P and its derivatives, DHP and THP, significantly improve the impaired sensitivity in diabetic rats. The heat sensitivity threshold is expressed as withdrawal latency in seconds and was evaluated at the beginning (A) and end of treatment (B) in control (C) and in diabetic rats treated following experimental protocol 2 with vehicle only (V), or with P, DHP, or THP. Data are mean±S.E.M. (number of rats in each group in parentheses). * $P<0.05$ vs. control; ** $P<0.01$ vs. control; # $P<0.05$ vs. diabetic injected with vehicle.

Skin biopsy confirmed the neuroprotective effect of neuroactive steroids (Fig. 8). Diabetes significantly reduced IENF density (45% compared with control rats; Fig. 9) and caused diffuse morphological changes of nerve fibers indicating axonal degeneration (Fig. 8). In diabetic rats, P improved IENF density, and DHP or THP totally restored it (Fig. 9).

DISCUSSION

Peripheral neuropathy is one of the most frequent and potentially severe complications of diabetes, leading to pain, skin ulcers, muscle weakness, loss of independence, and overall impairment of the patient's quality of life. Treatment of diabetic neuropathy is therefore a major goal but, despite multiple attempts, no satisfactory management is yet available. Neuroactive steroids are potential neuroprotective agents (Koenig et al., 1995; Melcangi et al., 2000, 2003; Azcoitia et al., 2003; Garcia-Segura et al., 2003; Schumacher et al., 2003; Sereda et al., 2003; Griffin et al., 2004; Garcia-Ovejero et al., 2005), but their use as therapeutic tools for the treatment of diabetic neuropathy has not received enough attention. Previous studies have shown that dehydroepiandrosterone prevents vascular and neuronal dysfunction in the sciatic nerve of STZ-rats (Yorek et al., 2002) and more recently we have observed that P and DHP may prevent morphological myelin alterations induced by diabetes (Veiga et al., 2006).

In this study, we show for the first time that STZ-induced diabetes causes P plasma levels to drop steeply in male rats, while both PREG and THP plasma levels were unmodified. This might be due to testicular and/or adrenal impairment. Namely, several observations have confirmed dysfunction in reproductive (i.e. neurohormones and steroid plasma levels) (El'tseva et al., 1993; Hassan et al., 1993; Babichev et al., 1998; Durant et al., 1998; Sudha et al., 2000; Tanaka et al., 2001; van Dam et al., 2003; Castellano et al., 2006; Salonia et al., 2006) and hypothalamo–pituitary–adrenal axis (Andreis et al., 1990; Rebuffat et al., 1990; Chan et al., 2001, 2002, 2005) associated with diabetes. We also found that chronic treatment with P restored normal plasma levels and had concomitant neuroprotective effects.

These observations raise some concern because elevated circulating levels of P might play a role in the development of gestational diabetes that occurs in 3–7% of pregnant women. In particular, P seems to accelerate the progression of diabetes in female *db*/*db* mice and knocking out the progesterone receptor (PR) has a beneficial effect on glucose clearance in female mice (Picard et al., 2002). However, although of potential interest, this concept remains controversial (Branisteanu and Mathieu, 2003; Margolis et al., 2004; Garris, 2005). In addition, P is actively converted into DHP and then into THP and these enzymatic conversions may have an important impact on the mechanism of action of these neuroactive steroids (Melcangi et al., 2001).

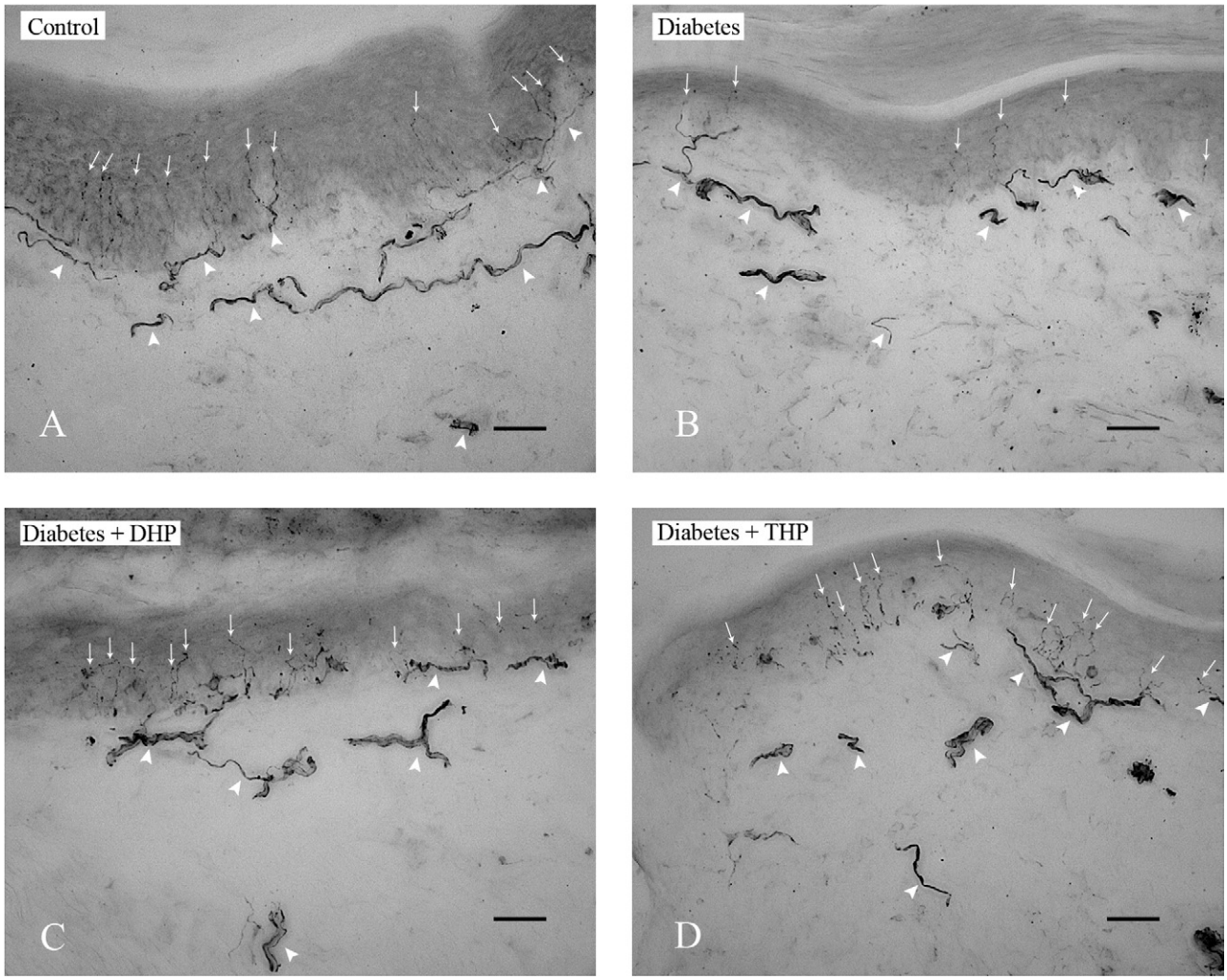

Fig. 8. Treatment with DHP or THP following experimental protocol 2 restores skin innervation in STZ-induced diabetic neuropathy. IENF density in the hindpaw footpad of control (A), diabetic treated with vehicle only (B), and diabetic rats treated following experimental protocol 2 with DHP (C) or THP (D). Microphotographs are bright-field immunohistochemistry with anti-PGP 9.5 antibodies in 20 μm-thick sections. Arrows indicate IENF; arrowheads indicate dermal nerve bundles (scale bar=30 μm). Note the marked reduction of IENF density and the weaker immunoreactivity to PGP 9.5 in diabetic rats, reflecting axonal degeneration. The density of IENF is completely recovered after treatment with DHP or with THP.

In our experimental paradigm, the STZ-induced model of diabetic neuropathy, chronic steroid treatment, besides raising plasma P levels, also counteracted the impairment of NCV and thermal threshold, restoring skin innervation density and myelin protein mRNA levels (i.e. P0 and PMP22), and improving of Na^+,K^+-ATPase activity. Interestingly, these beneficial effects were achieved not only by P, but also, by its derivatives DHP and THP. Moreover, we have observed that the schedule of treatment applied is also an important variable. Thus, we have detected an increased protective effect with a higher frequency and total amount of steroid administration. The administration of a total amount of 8 mg of steroids in injections of 1 mg every 4 days, an experimental protocol protective in other experimental models of peripheral neuropathy (Melcangi et al., 1998, 2000; Azcoitia et al., 2003), resulted in weaker protection in diabetic nerves than the administration of 16 mg of steroids in injections of 1 mg every 2 days. These findings indicate that, depending on the pathological insult, different doses and pattern of administration of neuroactive steroids may be required to protect peripheral nerves.

Low Na^+,K^+-ATPase activity in peripheral nerves is a potential mechanism to explain NCV slowing during hyperglycemia and we demonstrated that in human the reduction of Na^+,K^+-ATPase activity is not simply secondary to fiber loss, but quite likely contributes in the pathogenesis and self-maintenance of diabetic neuropathy (Scarpini et al., 1993). We also observed that Na^+,K^+-ATPase is sensitive to environmental hypoxia (Doss et al., 1997) and that pharmacological treatments able to prevent or restore this activity also protect or reverse the decrease in NCV (Bianchi et al., 2004). The present study takes these concepts further by demonstrating that in diabetic rats P and DHP significantly counteract the impairment of Na^+,K^+-ATPase activity and this, in agreement with other observations (Yorek et al., 2002), will possibly be a mechanism by which

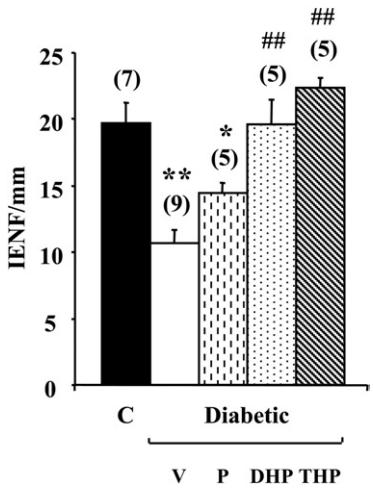

Fig. 9. Quantification of IENF density at the end of the experiment (12 weeks) in control (C) and diabetic rats treated following experimental protocol 2 with vehicle only (V), or with P, DHP, or THP. Data are expressed as the linear density of IEFN and are the mean±S.E.M. (number of rats in each group in parentheses). * $P<0.05$ vs. control; ** $P<0.01$ vs. control; ## $P<0.005$ vs. diabetic injected with vehicle.

they may improve neural function as assessed by NCV. The effect of neuroactive steroids on Na^+,K^+-ATPase activity may be mediated by several mechanisms, such as changes in the subcellular localization of the enzyme, in the expression or phosphorylation of the different Na^+,K^+-ATPase subunits or in Na^+ permeability in the axons. In addition, it is possible that the protection on Na^+,K^+-ATPase in diabetic rats by neuroactive steroids could be, at least in part, due to the improvement of oxidative stress as indicated by studies in STZ-diabetic rats treated with a naturally occurring steroids such as the dehydroepiandrosterone (Yorek et al., 2002). Intriguingly, P showed similar benefits in other models of neurodegeneration too. It counteracted the reduction of mRNA levels for alpha 3 catalytic and beta 1 regulatory subunits of neuronal ATPase after acute spinal cord transection in rat (Labombarda et al., 2002) and in a model of spinal cord neurodegeneration, the Wobbler mouse (Gonzalez Deniselle et al., 2002).

In agreement with these neurophysiologic benefits, treatment with P, DHP, or THP also significantly improved the hind-paw heat threshold in diabetic rats. Indeed, there are indications that P also reduces the sensitivity to pain by raising the levels of endorphins and opioid receptors in the CNS (Dawson-Basoa and Gintzler, 1997, 1998).

It has been recently demonstrated that neuropathic pain can affect neurosteroidogenesis. Sciatic nerve ligature increased the expression of cytochrome P450 side-chain cleavage (i.e. an enzyme that catalyzes cholesterol conversion into PREG) in spinal and supra-spinal networks and raised the concentrations of PREG and THP in the spinal cord (Patte-Mensah et al., 2004). Changes in the levels of neurosteroids in the spinal cord of STZ-diabetic rats (Saredi et al., 2005) have already been mentioned.

Since small fibers are deeply involved in thermal and nociceptive pathways, a reasonable correlate with the impairment of nociception in the hot plate test is the marked reduction in IENF density in rats with STZ-induced diabetic neuropathy. This is in agreement with previous findings in *db*/*db* and STZ-diabetic mice (Underwood et al., 2001). We suggest that the neuroactive steroids' protective effect against skin nerve fiber degeneration might explain the protection from thermal sensitivity impairment. Pathologic and behavioral findings correlated with neurophysiologic changes induced by diabetes and by neuroactive steroids, in agreement with a previous study in neurotoxic models of peripheral neuropathies (Lauria et al., 2005).

We also analyzed the effects of neuroactive steroids on mRNA levels of two important myelin proteins, P0 and PMP22, which play a crucial role in the maintenance of the multilamellar structure of peripheral nerve myelin (Bronstein, 2000; Suter and Scherer, 2003).

Treatment with P or DHP increased the low P0 mRNA levels in the sciatic nerve of STZ rats, but THP had no such effect. This difference is similar to what we previously observed in the model of experimental peripheral degeneration induced by nerve transection (Melcangi et al., 2000). Both P and DHP, but not THP, raised the low P0 mRNA levels in the distal stump of the transected nerve (Melcangi et al., 2000). In intact nerves, all these neuroactive steroids, including THP, stimulate the gene expression of P0 (Melcangi et al., 1998, 1999). Therefore, the lack of THP effect on myelin protein expression in nerve from diabetic rats might be explained by its different mechanism of action. In fact, while P and DHP interact with a classical steroid receptor like the PR (Melcangi et al., 2001), THP is a potent ligand of the GABA-A receptor (Lambert et al., 2003). In physiological condition, Schwann cells express both PR and GABA-A receptors (Melcangi et al., 1999; Magnaghi et al., 2001), but we have no information on their expression in diabetes. Hence, it is conceivable that diabetes may affect the ability of THP to interact with this receptor in Schwann cells.

From another point of view, it is also important to recall that the conversion of DHP into THP, by the enzyme 3alpha-hydroxysteroid dehydrogenase (3alpha-HSD), present in Schwann cells, is a reversible process (Melcangi et al., 2001). Thus, THP may be converted back to DHP and consequently exerts its effects through an interaction with PR. Either a loss of function or a deficiency of this enzymatic activity in diabetic nerves might therefore also be responsible for the lack of effect of THP on the expression of P0.

This lack of effect, due to changes in GABA-A receptors and/or 3alpha-HSD in Schwann cells, might be confirmed by the finding that THP, which in a non-pathological model stimulates the expression of PMP22 (Melcangi et al., 1999), is ineffective after nerve transection (Melcangi et al., 2000) like in the diabetic rat, as demonstrated here. In agreement with our recent results on Schwann cells from male rats (Magnaghi et al., 2006) and with observations obtained in different experimental conditions (Désarnaud et al., 1998), we show here that in diabetic rat P stimulates PMP22 gene expression, suggesting that this myelin protein too is sensitive to neuroactive steroids in this model.

In conclusion, our study shows that treatment with neuroactive steroids, such as P, DHP or THP, can decrease behavioral, neurophysiological, and pathological alterations induced by diabetes in a peripheral nerve. These findings are reinforced by the evidence that neuroactive steroids can usefully influence biochemical and functional parameters of peripheral nerves altered in diabetic neuropathy.

These effects seem to be mediated by different receptors. Thus, the expression of the myelin proteins considered so far and the Na^+,K^+-ATPase activity seem to be under the control of PR, while NCV, thermal nociceptive threshold and IENF density depend on both PR and GABA-A receptors. An important advancement for a possible therapeutic approach to diabetic neuropathy could be to act directly on classical (i.e. PR) and/or nonclassical steroid receptor (i.e. GABA-A) with specific synthetic ligands. A similar strategy might probably avoid the undesirable endocrine effects exerted by physiological neuroactive steroids.

For these reasons, future experiments will be extremely important to evaluate whether specific synthetic ligands of these classical and non-classical steroid receptors mimic the effects of P and its derivatives in counteracting neurodegenerative events in diabetes. Moreover, further studies of the potential benefits of neuroactive steroids in preventing diabetic neuropathy on antioxidant and vascular dysfunction are also needed.

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