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Neuroactive steroid treatment modulates myelin lipid profile in diabetic peripheral neuropathy

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ABSTRACT

Diabetic peripheral neuropathy causes a decrease in the levels of dihydroprogesterone and 5α-androstane-3α,17β-diol (3α-diol) in the peripheral nerves. These two neuroactive steroids exert protective effects, by mechanisms that still remain elusive. We have previously shown that the activation of Liver X Receptors improves the peripheral neuropathic phenotype in diabetic rats. This protective effect is accompanied by the restoration to control values of the levels of dihydroprogesterone and 3α-diol in peripheral nerves. In addition, activation of these receptors decreases peripheral myelin abnormalities by improving the lipid desaturation capacity, which is strongly blunted by diabetes, and ultimately restores the myelin lipid profile to non-diabetic values. On this basis, we here investigate whether dihydroprogesterone or 3α-diol may exert their protective effects by modulating the myelin lipid profile. We report that both neuroactive steroids act on the lipogenic gene expression profile in the sciatic nerve of diabetic rats, reducing the accumulation of myelin saturated fatty acids and promoting desaturation. These changes were associated with a reduction in myelin structural alterations. These findings provide evidence that dihydroprogesterone and 3α-diol are protective agents against diabetic peripheral neuropathy by regulating the *de novo* lipogenesis pathway, which positively influences myelin lipid profile.

1. Introduction

Diabetic peripheral neuropathy (DPN) is a common feature in patients with type 1 or type 2 diabetes. DPN is associated with myelin alterations, reduced nerve conduction velocity and increased thermal nociceptive response [1–4]. These deleterious effects in the peripheral nerves are also recapitulated in streptozotocin (STZ)-treated rats, a model of type 1 diabetes [5–11].

Neuroactive steroids are molecules from central and/or peripheral origin modulating neuronal and glial cell function by acting on steroid receptors or on GABA-A receptors [12–14]. Previous studies have shown that the levels of neuroactive steroids, like for instance progesterone (PROG), testosterone (T) and their metabolites, are reduced in the peripheral nerves of STZ diabetic rats [15,16]. In turn, the administration of metabolites of PROG or T, such as dihydroprogesterone (DHP) or 5α-androstane-3α,17β-diol (3α-diol) respectively, improves the neuropathic phenotype induced by diabetes [9–11]. A similar neuroprotective effect has been obtained with pharmacological tools able to increase neuroactive steroid levels in diabetic animals. Indeed ligands of Liver X Receptors (LXR), which are members of the nuclear receptor superfamily of transcription factors and regulate lipogenesis, promoting cholesterol utilization, restored to control values

Abbreviations: 3α-diol, 5α-androstane-3α,17β-diol; ACC, acetyl-CoA carboxylase α; AR, androgen receptor; DPN, diabetic peripheral neuropathy; DHP, dihydroprogesterone; ESI, electrospray ionization; FADS1 and FADS2, fatty acid desaturase 1 and 2; FAS, fatty acid synthase; GC–MS, gas chromatography mass spectrometry; IS, internal standards; LC–MS/MS, liquid chromatography tandem mass spectrometry; LXR, liver X receptors; P0, myelin glycoprotein zero; PMP22, peripheral myelin protein 22; PROG, progesterone; SIM, selective ion monitoring; SCD-1, stearoyl-CoA desaturase-1; SREBP-1c, sterol regulatory element binding protein-1c; STZ, streptozotocin; T, testosterone.

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the levels of DHP and 3α-diol in diabetic nerves [17,18]. These effects were associated with other changes in peripheral nerves, including decreased myelin structural alterations (*i.e.*, infoldings) and normalization of the myelin's lipid profile. This was mainly the consequence of the recovered expression of LXR classical target genes involved in fatty acid biosynthesis, primarily the lipogenic transcription factor Sterol Regulatory Element Binding Protein-1c (SREBP-1c), fatty acid synthase (FAS) and stearoyl-CoA desaturase-1 (SCD-1). In this context, we proved that diabetic animals were unable to properly desaturate fatty acids, resulting in an altered myelin lipid profile accounting for the myelin structural abnormalities (*i.e.*, infoldings). The LXR specific ligand ultimately ameliorates the neuropathic phenotype induced by diabetes by restoring the expression of genes involved in lipogenesis and, consequently, modifying the fatty acid species composition in myelin [8,17].

The experience gained with LXR activation on DPN together with the protective effects of neuroactive steroids so far ascertained [13,19] led us to evaluate whether DHP or 3α-diol may act on DPN by influencing myelin lipid profile, through the regulation of fatty acid biosynthetic pathway and ultimately protecting myelin structure and function.

To this aim, the effect of the treatment with DHP or 3α-diol has been evaluated in the sciatic nerve of STZ-treated rats analyzing myelin lipid profile by liquid chromatography tandem mass spectrometry (LC–MS/MS) and gas chromatography mass spectrometry (GC–MS). The lipidomic profile was corroborated by gene expression analysis of key genes in fatty acid biosynthesis and was complemented by a morphometric analysis of myelinated fibers.

2. Materials and methods

2.1. Animals

All experiments were conducted strictly following the regulations of the European Union (Directives 1986/609/EEC and 2010/63/UE, Official Journal L 358, 18/12/1986 pp. 0001–0028 and L 276, 20/10/2010, pp. 33–79, respectively) and local regulations (Italian Legislative Decree n. 116—27/01/1992) for the care and use of laboratory animals. Animal protocol was approved by the Institutional Review Committee. Diabetes was induced in 2-month-old male Sprague-Dawley rats (Crl:CD BR, Charles River, Lecco, Italy) by a single intraperitoneal injection of streptozotocin (STZ) 65 mg/kg (Sigma-Aldrich, Milano, Italy) in 0.09 M citrate buffer pH 4.8 as previously described [18]. Hyperglycemia was verified after 2 days, and rats with blood glucose concentrations >300 mg/dl were included in the study. Two months after the induction of diabetes, animals were treated every other day and for a month with a subcutaneous injection of DHP or 3α-diol (3.3 mg/kg body weight; total of sixteen treatments). The treatment schedule applied was the same previously demonstrated by us to exert neuroprotective effects in diabetic neuropathy [9,10]. Non-diabetic and diabetic control rats were treated with vehicle (sesame oil). Special care was taken to minimize animal suffering and to reduce the number of animals used to the minimum required for statistical accuracy.

2.2. Thermal nociceptive threshold

This neurophysiological parameter was assessed throughout the three months of diabetes to evaluate development of peripheral neuropathy and the effects of neuroactive steroids [18]. The test was done in a temperature-controlled room adjacent to the animals' housing room and animals' vital parameters were monitored.

2.3. Myelin purification and extraction of total fatty acids and cholesterol

The internal standards (IS) heneicosanoic acid (C21:0), linoleic acid-^{13}C (C18:2) and 5α-cholestane, and all other standards for fatty acids and cholesterol analyses were purchased from Sigma-Aldrich (Milano, Italy).

Myelin was purified from sciatic nerve as previously described [20]. IS were added to myelin samples and lipid extraction was performed with methanol (MeOH). The extract was splitted in two fractions: one for the analysis of free cholesterol (fraction A), and the other for the analysis of total fatty acids (fraction B). Total fatty acids were obtained by acid hydrolysis [21]. Briefly, fraction B was dissolved in chloroform/MeOH 1:1 v/v. Then, 1 M HCl:MeOH (1:1, v/v) solution was added to the total lipid extract and the mixed solution was shaken for 2 h. Then, chloroform:water (1:1 v/v) was added and the organic phase was collected and dried under nitrogen flow. The residue was dissolved in 500 μl of MeOH.

2.4. Lipidomic profile by liquid chromatography-tandem mass spectrometry (LC–MS/MS)

For fatty acid quantification, aliquots of each sample (10 μl) were diluted 1:10 in MeOH/water (50:50 v/v), transferred into a 96 well plate and placed in an auto-sampler for LC–MS/MS analysis. Quantitative analysis was performed with calibration curves prepared and analyzed daily by electrospray ionization (ESI) using an API 4000 triple quadrupole instrument (AB Sciex, USA). The LC mobile phases were: water/10 mM isopropylethylamine/15 mM acetic acid (phase A) and MeOH (phase B). The gradient (flow rate 0.5 ml/min) was as follows: T 0: 20% A, T 20: 1% A, T 25: 1% A, T 25.1: 20% A, T 30: 20% A. The Hypersil GOLD C8 column (100 mm × 3 mm, 3 μm) was maintained at 40 °C. The mass spectrometer was operated in selective ion monitoring (SIM)/SIM mode.

2.5. Myelin free cholesterol quantification

Fraction A for the quantitative analyses of free cholesterol, was first derivatized with a mixture of bis-trimethylsilyltrifluoroacetamide (BSTFA):pyridine (4:1 v/v) for 30 min at 60 °C, and then injected into a gas chromatograph–mass spectrometer (GC–MS, Varian Saturn 2100). The MS was operated in the electron impact ionization mode. GC–MS analysis was performed as follows: 1 μl sample was injected in splitless mode (inlet was kept at 270 °C with the helium flow at 1.0 ml/min) at the initial 180 °C. The oven was first kept at 180 °C for 1 min, ramped at 50 °C/min to 240 °C, then at 5 °C/min to 300 °C for 6 min. The ions used for the quantification of cholesterol were at *m/z* 368 for cholesterol and *m/z* 357 for 5α-cholestane, the IS. The selection of ions for SIM analysis was based on mass spectra of pure standards and the quantification was based on calibration curves freshly prepared in a concentration range from 0 to 10 μg/μl. Cholesterol esters were evaluated to verify the myelin quality and purity using the aliquots of samples that underwent acid hydrolysis.

2.6. Quantitative real time PCR (RT-qPCR)

RNA was prepared by using Nucleospin® RNA II kit (Macherey-Nagel, Milano, Italy). RNA was analyzed by TaqMan CFX384 RT-qPCR using the iScriptTM one-step RT-PCR kit for probes (Bio-Rad, Milano, Italy). Samples were arrayed in 384-well format in triplicate