Neuroscience 199 (2011) 401–409

SEX-DIMORPHIC EFFECTS OF DEHYDROEPIANDROSTERONE IN DIABETIC NEUROPATHY

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Abstract—Our recent observations have demonstrated that gonadectomy in female, but not in male diabetic animals, exert protection in the peripheral nervous system and that these effects were associated with an increase in the levels of dehydroepiandrosterone (DHEA) in the female sciatic nerve [Pesaresi M, Giatti S, Cavaletti G, Abbiati F, Calabrese D, Bianchi R, Caruso D, Garcia-Segura LM, Melcangi RC (2011) Exp Neurol 228:215–221]. That is interesting because the neuroprotective effects of this neuroactive steroid have so far only been analyzed in male diabetic animals. Using the experimental model of streptozotocin-induced diabetic neuropathy, we have here compared the effect of DHEA treatment in male and in female animals. Data obtained indicate that DHEA treatment is able to counteract the decrease in nerve conduction velocity (NCV) induced by diabetes in both sexes. However, it was only in females that this neuroactive steroid was able to reestablish NCV to control levels. In addition, it was only in females that DHEA exerted neuroprotective actions on functional (i.e., thermal sensitivity) or molecular parameters, such as gene expression of myelin proteins. Sex-depending neuroprotective effects of DHEA were also confirmed by the finding that it was only in females that this neuroactive steroid fully restored the intra-epidermal nerve fiber density, which was decreased by diabetes. Interestingly, the metabolic fate of DHEA is also different in males and females. Thus, analysis of the neuroactive steroid levels after the treatment with DHEA indicates that in the sciatic nerve of male diabetic animals 17α-estradiol levels decrease in association with an increase of its isomer 17β-estradiol and with a decrease in the levels of α-androstane-3α, 17β-diol. These changes were not observed in the sciatic nerve of females. Altogether, these results suggest that DHEA could be considered as a candidate for a sex-specific therapy based on neuroactive steroids.

Key words: liquid chromatography-tandem mass spectrometry, neuroactive steroids, myelin proteins, peripheral nerve, rat, streptozotocin.

Peripheral neuropathy is an important complication of diabetes. Current therapeutic strategy relies on the control of glycemic and vascular risk factors, which however does not totally prevent its occurrence and progression. Recent results, obtained in the experimental model of streptozotocin (STZ)-induced neuropathy, have shown that neuroactive steroids might represent an interesting therapeutic option (Roglio et al., 2008). In fact, as demonstrated by different laboratories, progesterone (PROG), testosterone (T), and their derivatives, as well as dehydroepiandrosterone (DHEA), exert in STZ experimental model neuroprotective effects at biochemical, functional, neurophysiological, and neuropathological levels (Yorek et al., 2002; Veiga et al., 2006; Leonelli et al., 2007; Roglio et al., 2007). For instance, chronic treatment for 1 month with PROG, or with its derivatives, dihydroprogesterone (DHP) and tetrahydroprogesterone (THP), counteracted the impairment of nerve conduction velocity (NCV) and thermal threshold, restored skin innervation density of the hindpaw footpad, improved Na^+,K^+-ATPase activity, and increased mRNA levels of myelin proteins, such as glycoprotein zero (P0) and peripheral myelin protein 22 (PMP22) (Leonelli et al., 2007). PROG and DHP are also able to counteract the increase in the number of fibers with myelin infoldings induced by STZ treatment in the sciatic nerve (Veiga et al., 2006). Similarly, T derivatives, such as dihydrotestosterone (DHT) or 5α -androstan-3α, 17β -diol (3α-diol), increased tail NCV, partially counteracted the increase of thermal threshold, and reversed the reduction of intraepidermal nerve fiber (IENF) density induced by diabetes. In addition, treatment with DHT increased tibial Na^+,K^+-ATPase activity and the expression of myelin protein P0 in the sciatic nerve (Roglio et al., 2007). Furthermore, DHEA prevents vascular and neuronal dysfunction in the sciatic nerve of STZ rats (Yorek et al., 2002). In agreement with the protective effect of neuroactive steroids, pharmacological tools able to increase their levels in peripheral nerves

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Abbreviations: 3α-diol, α-androstane-3α, 17β-diol; 3β-diol, α-androstane-3β, 17β-diol; 17α-E, α-estradiol; 17β-E, β-estradiol; D9-PROG, 2,2,4,6,6-17α,21,21,21-D9-PROG; ANOVA, analysis of variance; APCI, atmospheric pressure chemical ionization; AR, androgen receptor; DHEA, dehydroepiandrosterone; DHP, dihydroprogesterone; DHT, dihydrotestosterone; IENF, intra-epidermal nerve fiber; LC-MS/MS, liquid chromatography-tandem mass spectrometry; MAL, myelin and lymphocyte-associated protein; NCV, nerve conduction velocity; P0, glycoprotein zero; PMP22, peripheral myelin protein 22; PROG, progesterone; STZ, streptozotocin; T, testosterone; THP, tetrahydroprogesterone.

0306-4522/11 $ - see front matter
doi:10.1016/j.neuroscience.2011.09.013

are also neuroprotective in this experimental model (Giatti et al., 2009; Cermenati et al., 2010). Our recent studies have suggested that changes of hormonal milieu induce biochemical and/or cellular changes in peripheral nerve that render the tissue more resistant to the damage induced by diabetes (Pesaresi et al., 2011). This is a sexdimorphic effect, because in ovariectomized rats, diabetes does not result in the significant modifications on NCV, Na^+,K^+-ATPase activity, and expression of P0 and PMP22 myelin proteins observed in intact diabetic females, whereas diabetes induced similar changes in these parameters in orchidectomized and intact males. Interestingly, protective effects exerted by ovariectomy are associated with an increase in the levels of DHEA, T, and DHT in the sciatic nerve, with no changes in the levels of these neuroactive steroids in plasma (Pesaresi et al., 2011). Therefore, these findings suggest that in case of diabetic neuropathy, female animals are more sensitive to specific neuroactive steroids.

As mentioned previously, DHEA exerts neuroprotective effects on diabetic neuropathy counteracting the alterations in NCV and Na^+K^+-ATPase activity (Yorek et al., 2002). However, the protective effects of DHEA have so far only been assessed in males; on this basis, we have here analyzed whether DHEA treatment may also be neuroprotective in diabetic females. In particular, the effect of this neuroactive steroid has been analyzed on NCV, thermal sensitivity, IENF density, Na^+,K^+-ATPase enzymatic activity, and gene expression of myelin proteins, such as P0, PMP22, and myelin and lymphocyte-associated protein (MAL) in female and male diabetic animals.

EXPERIMENTAL PROCEDURES

DHEA, testosterone, 5α-androstane-17β-ol-3-one (DHT), 3α-diol, 5α androstane-3β, 17β-diol (3β-diol), 17α-estradiol (17α-E), and 17β-estradiol (17β-E) were purchased from Sigma Aldrich, Milano, Italy. 2,2,4,6,6–17α,21,21,21-D9-PROG (D9-PROG) was obtained from Medical Isotope (Pelham, NH, USA); 2,4,16,16-D4-17β-estradiol (D4-17β-E) was obtained from CDN Isotope Pointe Claire (QuebecCanada). SPE cartridges (Discovery DS-C18 500 mg) were from Supelco, Italy. All solvents and reagents were HPLC grade (Sigma Aldrich, Italy).

Animals

Two-month-old male and female Sprague–Dawley rats, Crl: CD BR (Charles River, Calco, Italy), were used. The animals were housed in the animal care facility of the Department of Endocrinology, Pathophysiology and Applied Biology at the University of Milan with controlled temperature and humidity. The light schedule was 14 h light and 10 h dark (lights on at 6.30 h). The animals were handled following the European Union Normatives (Council Directives 86/609/EEC and 2010/63/UE), 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.

Induction of diabetes

Diabetes was induced by a single i.p. injection of freshly prepared STZ (60 mg/kg; Sigma, Italy) in citrate buffer 0.09 M pH 4.8 (Leonelli et al., 2007). Control animals were injected with 0.09 M citrate buffer at pH 4.8. Hyperglycemia was confirmed 48 h after STZ injection by measurement of tail-vein blood glucose levels using a Glucomen tester (Menarini, Florence, Italy). Only animals with mean plasma glucose levels above 300 mg/dl were classified as diabetic. Glycemia was also confirmed before the treatment and tested at scheduled death, 3 months after STZ.

At 2 months after the STZ injection, rats received 16 s.c. injections (every other day) of DHEA (Sigma, Italy, 2.8 mg/kg) dissolved in 200 μl sesame oil. Control rats received 200 μl vehicle (sesame oil). Rats were killed 24 h after the last treatment.

Tail NCV

NCV in the tail was measured by using a Myto EBNeuro electromyography (EBNeuro, Firenze, Italy) as previously described (Tredici et al., 1998). Briefly, the antidromic NCV in the tail nerve was assessed by placing recording ring electrodes distally on the tail, whereas the stimulating ring electrodes were placed 5 and 10 cm proximally from 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 determinations were done under standard conditions in a temperature-controlled room.

Na^+,K^+-ATPase activity

Tibial stumps were dissected out, desheathed, 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 Potter-Elvehjem 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.

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±0.2 °C. 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 2 weeks starting from the second week after STZ injection. Animals were tested twice, with a 30-min interval, in each test section.

Skin biopsies

Peripheral nerve damage was assessed by the quantification of the IENF density in the skin of the hindpaw footpad (Lauria et al.,

Table 1. Body weight and glucose levels

Animal	Body weight (g)	Body weight (g)	Blood glucose (mg/100 ml)
	Before STZ injection	At sacrifice	At sacrifice
M Ctrl	343.8±2.2	548.9±13.7	106±3
M Diabetic	350.6±1.9	309.6±11.9***	505±2***
M DHEA	342.8±2.4	321.9±11.7***	588±2***
F Ctrl	230.4±5.9	297.9±6.9	109±4
F Diabetic	224.2±8.7	202.2±19.8*	577±4***
F DHEA	224.1±6.4	204.9±15.1*	576±3***

Data are expressed as mean±SEM (n=10). * $P<0.05$ and *** $P<0.001$ vs. Ctrl.