Neurobiology of Disease

Activation of the Liver X Receptor Increases Neuroactive Steroid Levels and Protects from Diabetes-Induced Peripheral Neuropathy

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Neuroactive steroids act in the peripheral nervous system as physiological regulators and as protective agents for acquired or inherited peripheral neuropathy. In recent years, modulation of neuroactive steroids levels has been studied as a potential therapeutic approach to protect peripheral nerves from damage induced by diabetes. Nuclear receptors of the liver X receptor (LXR) family regulate adrenal steroidogenesis via their ability to control cholesterol homeostasis. Here we show that rat sciatic nerve expresses both LRXα and β isoforms and that these receptors are functional. Activation of liver X receptors using a synthetic ligand results in increased levels of neurosteroids and protection of the sciatic nerve from neuropathy induced by diabetes. LXR ligand treatment of streptozotocin-treated rats increases expression in the sciatic nerve of steroidogenic acute regulatory protein (a molecule involved in the transfer of cholesterol into mitochondria), of the enzyme P450scc (responsible for conversion of cholesterol into pregnenolone), of 5α-reductase (an enzyme involved in the generation of neuroactive steroids) and of classical LXR targets involved in cholesterol efflux, such as ABCA1 and ABCG1. These effects were associated with increased levels of neuroactive steroids (e.g., pregnenolone, progesterone, dihydroprogesterone and 3α-diol) in the sciatic nerve, and with neuroprotective effects on thermal nociceptive activity, nerve conduction velocity, and Na^+, K^+-ATPase activity. These results suggest that LXR activation may represent a new pharmacological avenue to increase local neuroactive steroid levels that exert neuroprotective effects in diabetic neuropathy.

Introduction

Liver X receptors (LXRs) are ligand activated transcription factors that belong to the nuclear receptor superfamily. Two different isoforms, LXRα (NR1H3) and LXRβ (NR1H2) are known. LXRα is predominantly expressed in liver and at lower levels in intestine, macrophages, adipose tissue, lung, kidney and adrenal gland; LXRβ is broadly expressed (Li et al., 2004). The LXRs serve as cholesterol sensors that prevent excessive intracellular accumulation of cholesterol. Oxysterols (oxidized forms of cholesterol) activate the LXRs and induce expression of a battery of genes aimed at eliminating harmful concentrations of cholesterol, including genes involved in cholesterol efflux such as the ATP binding cassette family of transporters (e.g., ABCA1, ABCG1) (Beaven and Tontonoz, 2006; Cummins and Mangelsdorf, 2006).

Recently, it was reported that LXRs directly modulate steroidogenic acute regulatory protein (StAR) expression in the adrenal gland, a transfer protein regulating cholesterol shuttling into mitochondria, a key step in the initiation of steroid hormone synthesis (Cummins et al., 2006). Steroidogenesis also occurs in peripheral nerves where it results in the formation of neuroactive steroids (Garcia-Segura and Melcangi, 2006; Melcangi and Mensah-Nyagan, 2008; Roglio et al., 2008a). Peripheral nerves express proteins involved in the initiation of steroidogenesis, such as StAR and the translocator protein-18 kDa (TSPO), as well as the enzymes involved in the synthesis and metabolism of neuroactive steroids, such as cytochrome P450 side chain cleavage (P450scc), 3β-hydroxysteroid dehydrogenase, and 5α-reductase (5α-R) (Garcia-Segura and Melcangi, 2006; Melcangi et al., 2008;

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Roglio et al., 2008a). Neuroactive steroids act in peripheral nerves as physiological regulators and as protective agents for acquired and inherited peripheral neuropathy (Melcangi and Garcia-Segura, 2006; Schumacher et al., 2007; Roglio et al., 2008b; Melcangi and Panzica, 2009). This suggests that increasing the levels of neuroactive steroids directly in the nervous system could be a therapeutic approach that may avoid the potential endocrine side effects of systemic administration of neuroactive steroids. Indeed, we recently reported that a ligand of TSPO (Ro5-4864) that increases the local concentration of neuroactive steroids is effective at reducing the severity of diabetic neuropathy (Giatti et al., 2009).

Because the LXRs control steroidogenesis in other settings, here we have used the experimental model of streptozotocin (STZ)-induced diabetic neuropathy to evaluate whether treatment with synthetic LXR ligands (e.g., GW3965) is able to increase neuroactive steroid levels and exert neuroprotective effects. As described by Collins and coworkers (Collins et al., 2002), GW3965 binds and activates specifically LXRα and LXRβ, and no other nuclear receptors. GW3965 is a chemical tool widely used to understand the biology of the LXRs. We find that LXR activation drives neuroactive steroid synthesis in sciatic nerve, and that treatment with LXR ligands ameliorates diabetes-induced neuropathy, as judged by a variety of functional and biochemical parameters.

Materials and Methods

Reagents. 5-Pregnen-3β-ol-20-one (PREG), progesterone (PROG), 5α-pregnane-3, 20-dione (DHP), 3α-hydroxy-5α-pregnen-20-one (THP), 3β-hydroxy-5α-pregnen-20-one (isopregnanolone), testosterone (T), 5α-androstane-17β-ol-3-one (DHT), 5α-androstane-3α,17β-diol (3α-diol), and dehydroepiandrosterone (DHEA) were purchased from Sigma-Aldrich. 2,2,4,6,6–17α,21,21,21-D_9-PROG (D_9-PROG) was purchased from Medical Isotopes; 2,4,16,16-D_4-17β-estradiol (D_4-17β-E) was obtained from CDN Isotopes. 17,21,21,21-D_4-PREG (D_4-PREG) was kindly synthesized by Prof. P. Ferraboschi (Department of Medical Chemistry, Biochemistry and Biotechnology, Universitá di Milano, Milano, Italy). Solid phase extraction (SPE) cartridges (Discovery DS-C18 500 mg) were from Supelco. All solvents and reagents were HPLC grade (Sigma-Aldrich). GW3965 was synthesized in house as previously described (Marino et al., 2009).

Animals. Two-month-old male Sprague Dawley rats, Crl:CD BR (Charles River) were used. The animals were maintained in quarters with controlled temperature and humidity. The light schedule was 14 h light and 10 h dark (lights on at 6:30 AM). The animals were handled following the European Union Normative (Council Directive 86/609/EEC), with the approval of the 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 and experimental treatments. Diabetes was induced by a single i.p. injection of freshly prepared streptozotocin (65 mg/kg; Sigma) in 0.09 M citrate buffer, pH 4.8. Control animals were injected with 0.09 M citrate buffer at pH 4.8. Hyperglycemia was confirmed 48 h after streptozotocin injection by measuring tail vein blood glucose levels using a glucometer OneTouch Ultra2 (LifeScan). Only animals with mean plasma glucose levels >300 mg/ml were classified as diabetic. Glycemia was also assessed before treatment with GW3965 (2 months after streptozotocin injection, see below) and before death, 3 months after streptozotocin administration. At 2 months, diabetic animals were treated once a week with GW3965 50 mg/kg (i.e., they received 4 subcutaneous injections). The rationale for the once a week treatment is that daily systemic activation of LXRs is thought to result in hypertriglyceridemia (due to increased hepatic lipogenesis), an undesirable side effect (Schultz et al., 2000). Thus, we sought to use the minimal dose of GW3965 that would show benefit while minimizing the potential for side effects.

Control rats received 200 μl of vehicle (sesame oil). Rats were killed 24 h after the last treatment.

Assessment of neuroactive steroids by liquid chromatography–tandem mass spectrometry. Samples were extracted and purified according to (Caruso et al., 2008). Briefly, samples (100 mg/tissue) were added with internal standards and homogenized in 2 ml of MeOH/acetic acid (99:1, v/v) using an ultrasonic homogenizer (Bransonic Ultrasonics). After an overnight incubation at 4°C, samples were centrifuged at 12,000 rpm for 5 min and the pellet was extracted twice with 1 ml of MeOH/acetic acid (99:1, v/v). The organic phases were combined and dried with a gentle stream of nitrogen in a 40°C water bath. Samples were resuspended with 3 ml of MeOH/H_2O (10:90, v/v) and passed through an SPE cartridge, previously activated with MeOH (5 ml) and MeOH:H_2O 10:90 (v/v) (5 ml). Steroids were eluted in MeOH, concentrated and transferred into auto-sampler vials before liquid chromatography–tandem mass spectrometry (LC–MS/MS) analysis. Quantitative analysis was performed on the basis of calibration curves prepared and analyzed using deuterated internal standards. Calibration curves were extracted and analyzed as described above for samples.

Positive atmospheric pressure chemical ionization (APCI) experiments were performed using a linear ion trap-mass spectrometer (LTQ, Thermo Electron) equipped with a Surveyor liquid chromatography Pump Plus and a Surveyor Autosampler Plus (Thermo Electron). The LC mobile phases were (A) H_2O/0.1% formic acid and (B) methanol (MeOH)/0.1% formic acid. The gradient (flow rate 0.5 ml/min) was as follows: T0.0 70% A, T1.5 70% A, T2.0 55% A, T3.0 55% A, T35.0 36% A, T40.0 25% A, T41.0 1% A, T45.0 1% A, T45.2 70% A, T55.0 70% A. The split valve was set at 0–6.99 min to waste, 6.99–43.93 min to source and 43.93–55 to waste. The Hypersil Gold column (100 × 3 mm, 3 μm; Thermo Electron) was maintained at 40°C. The injection volume was 25 μl and the injector needle was washed with MeOH/ water 1/1 (v/v). Peaks of the LC–MS/MS were evaluated using a Dell workstation by means of the software Excalibur release 2.0 SR2 (Thermo Electron).

The mass spectrometer was operated in the positive ion mode with the APCI source using nitrogen as sheath, auxiliary and sweep gas at flow rates of 23, 8, 2 (arbitrary units), respectively. Other ion-source parameters: vaporizer temperature 450°C, ion-source collision-energy (SID) 20 V, capillary temperature 275°C. The mass spectrometer was used in MS/MS mode using helium as collision gas. The relative collision-energy was set at 35% for D_4-17β-E, 3α-diol and at 35% using the Wide Band Activation mode (Thermo Electron) for all the other steroids. Samples were analyzed using the transitions previously reported by (Pesaresi et al., 2010).

Thermal nociceptive threshold. 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 hindpaw, 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 between tests.

Nerve conduction velocity. At the end of treatment, antidromic tail NCV was assessed using a Myto EBNeuro electromyography apparatus as previously described (Tredici et al., 1998; Meregalli et al., 2010). Briefly, recording ring electrodes were placed distally in the tail of unanesthetized animals. The stimulating ring electrodes were placed 5 and 10 cm proximally with respect to the recording point. Latency of the potentials recorded at the two sites after nerve stimulation was determined (peak-to-peak, stimulus duration 100 ms, filter 1 Hz–5 MHz) and NCV calculated. All neurophysiological studies were done under standard conditions in a temperature-controlled room adjacent to the animal housing room. Body temperature and vital conditions of the animals were monitored during the neurophysiological examination.

Na^+, K^+-ATPase activity. Tibial stumps were dissected out, desheathed and homogenized in a 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 Elvehjem–Potter homogenizer (DISA), and stored at −80°C for ATPase determinations. Na^+, K^+-ATPase activity was determined

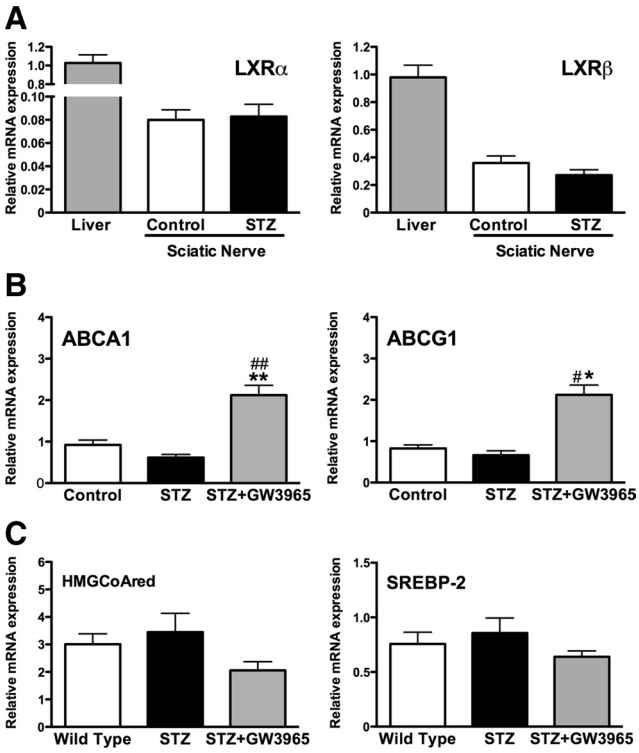

Figure 1. ***A***, LXRα and LXRβ are expressed in sciatic nerve and their levels are unchanged between control and STZ-treated rats. ***B***, LXR activation by GW3965 treatment induces mRNA levels of ABCA1 and ABCG1, classical LXR target genes involved in cholesterol efflux in the sciatic nerve. These data indicate that the ligand reaches the sciatic nerve and that the LXRs are activated. ***C***, Expression levels of HMGCoA reductase and SREBP-2, two genes involved in cholesterol synthesis. The mRNA levels of these genes are unchanged by diabetes and/or by GW3965 treatment. The bars represent the relative mRNA expression of shown genes to the housekeeping gene 36B4. Data are presented as mean ± SEM ($n = 9$). Statistical analysis is performed by one-way ANOVA followed by Tukey–Kramer posttest. $^{*}p < 0.05$, $^{**}p < 0.001$ vs control rats; $^{\#}p < 0.05$, $^{\#\#}p < 0.001$ vs STZ-treated rats.

spectrophotometrically as previously described (Bianchi et al., 2004). Protein content in homogenates was determined by Lowry's method with bovine serum albumin as standard.

Real-time PCR. RNA was prepared using the Nucleospin RNA II kit (Macherey-Nagel). RNA was analyzed by a TaqMan qRT-PCR (quantitative real-time) instrument (CFX384 real time system, Bio-Rad Laboratories) using the iScriptTM one-step RT-PCR kit for probes (Bio-Rad Laboratories). Samples were run in 384 well formats in triplicate as multiplexed reactions with a normalizing internal control (36B4). Probe and primer sequences were purchased from Eurofins MWG-Operon, and are available on request.

Statistical analysis. Quantitative data were analyzed through statistical analysis according with the experimental protocols and the nature of the data. Data from experiments with more than two variables were analyzed by one-way ANOVA followed by Tukey–Kramer posttest. A $p \leq 0.05$ indicates a statistically significant effect. All statistical analyses were performed in GraphPad PRISM (version 5).

Results

The LXRs are active in sciatic nerve

To investigate the potential role of LXRs in diabetes-induced peripheral neuropathy we first assessed whether the two isoforms of LXR are expressed in a peripheral nerve, such as the rat sciatic nerve, and whether their levels change in diabetic animals. LXRα and LXRβ are both expressed in rat sciatic nerve and their mRNA levels are unaffected by diabetes (Fig. 1A). LXRβ is the dominant isoform present in sciatic nerve. The expression of LXRα and LXRβ in sciatic nerve is substantially lower than that in liver, a tissue with considerable LXR activity. To establish whether the levels of LXR present in sciatic nerve are of functional significance, we measured LXR target gene expression after administration of a synthetic LXR ligand. GW3965 was given once a week for a month to STZ-treated rats 2 months after the induction of diabetes. Expression of two *bona fide* LXR target genes involved in cholesterol efflux (ABCA1 and ABCG1), which are not regulated in sciatic nerve in this model of diabetes, was significantly increased after treatment with GW3965 (Fig. 1B), indicating that LXRs are active transcriptional regulators in sciatic nerve. In contrast, GW3965 treatment did not affect expression of key regulators of cholesterol synthesis, such as HMGCoA reductase and SREBP-2 (Fig. 1 C).

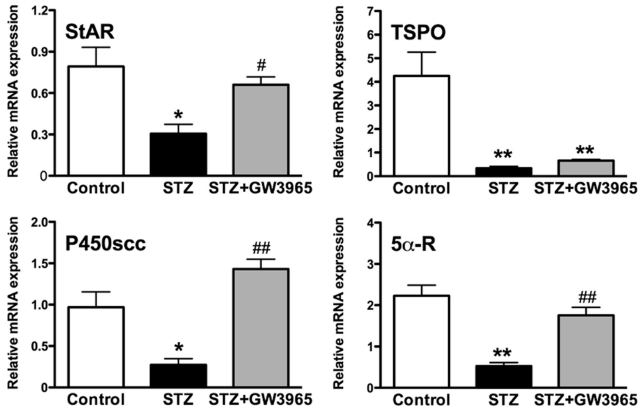

Figure 2. Gene expression of steroidogenic acute regulatory protein (StAR), translocator protein-18 kDa (TSPO), cytochrome P450 side chain cleavage (P450scc) and 5α-reductase (5α-R), in sciatic nerve. As shown, LXR activation by GW3965 treatment in diabetic rats restores to normal levels the expression of StAR, P450scc and 5α-R but it does not affect TSPO levels. The bars represent the relative mRNA expression of shown genes to the housekeeping gene 36B4. Data are presented as mean ± SEM ($n = 9$). Statistical analysis is performed by one-way ANOVA followed by Tukey–Kramer posttest. $^{*}p < 0.05$, $^{**}p < 0.001$ vs control rats; $^{\#}p < 0.05$, $^{\#\#}p < 0.001$ vs STZ-treated rats.

Activation of LXR modulates neuroactive steroid levels

Compared with normoglycemic controls, diabetic rats showed notable differences in expression of several important genes involved in steroidogenesis and neuroactive steroid metabolism. In particular, mRNA levels of StAR and TSPO (molecules involved in cholesterol shuttling into the mitochondria), P450scc (the enzyme responsible of the conversion of cholesterol into pregnenolone), and 5α-R (which converts progesterone and testosterone into their 5α-reduced metabolites, dihydroprogesterone and dihydrotestosterone) were significantly decreased in the diabetic state (Fig. 2). Treatment of diabetic animals with an LXR activator restored mRNA levels of StAR, P450scc and 5α-R to the level seen in normoglycemic controls, but had no effect on TSPO expression (Fig. 2). In agreement with these gene expression patterns, we observed that diabetes decreased neuroactive steroid levels in sciatic nerve, and that treatment with an LXR ligand counteracted these effects. Indeed, LC–MS/MS analysis showed that the levels of PREG, PROG, DHP, isopregnanolone, T and its derivatives, DHT and 5α-androstane-3α,17β-diol (3α-diol) were significantly decreased in the sciatic nerve of diabetic rats (Table 1). LXR activation completely reversed the diabetes-induced decrease in PREG, PROG, DHP and 3α-diol levels. Interestingly, the levels of these neuroactive steroids also fell in the plasma of diabetic rats, but treatment with the LXR agonist had no effect on them (Table 2).

Table 1. Analysis of neuroactive steroid levels by LC–MS/MS in sciatic nerves of control, STZ, and STZ rats treated with GW3965

	PREG	PROG	DHP	THP	Isopregnanolone	DHEA	T	DHT	3α-diol
Control	1.52 ± 0.23	1.58 ± 0.22	8.86 ± 0.59	1.00 ± 0.16	2.19 ± 0.38	0.24 ± 0.04	1.52 ± 0.23	0.80 ± 0.11	1.01 ± 0.13
STZ	0.63 ± 0.05*	0.62 ± 0.09*	5.89 ± 0.24**	0.65 ± 0.03	0.61 ± 0.07**	0.17 ± 0.02	0.24 ± 0.05**	0.18 ± 0.03**	0.57 ± 0.10*
STZ + GW3965	1.45 ± 0.33#	1.71 ± 0.24##	9.17 ± 0.59##	0.92 ± 0.05	1.07 ± 0.06*	0.29 ± 0.04	0.27 ± 0.13**	0.20 ± 0.04**	1.09 ± 0.13#

Data are expressed as pg/mg of tissue and are represented by mean ± SEM. Control, $n = 8$; STZ, $n = 8$; STZ + GW3965, $n = 6$. Statistical analysis was performed by one-way ANOVA followed by Tukey–Kramer posttest. *$p < 0.05$, **$p < 0.001$ versus control rats; #$p < 0.05$, ##$p < 0.001$ versus STZ-treated rats.

Table 2. Analysis of neuroactive steroids levels by LC–MS/MS in plasma of control, STZ, and STZ rats treated with GW3965

	PREG	PROG	DHP	THP	Isopregnanolone	DHEA	T	DHT	3α-diol
Control	0.64 ± 0.07	0.82 ± 0.12	1.22 ± 0.11	0.49 ± 0.10	0.33 ± 0.10	0.059 ± 0.007	4.12 ± 0.57	0.065 ± 0.006	1.63 ± 0.27
STZ	0.32 ± 0.09*	0.28 ± 0.05**	0.83 ± 0.04*	0.36 ± 0.12	0.28 ± 0.09	0.058 ± 0.009	0.70 ± 0.021**	0.064 ± 0.01	0.54 ± 0.13**
STZ + GW3965	0.29 ± 0.03*	0.68 ± 0.19	0.78 ± 0.16*	0.75 ± 0.17	0.68 ± 0.19	0.065 ± 0.008	0.25 ± 0.08**	0.055 ± 0.005	0.71 ± 0.21*

Data are expressed as pg/μl of plasma and are represented by mean ± SEM. Control, $n = 8$; STZ, $n = 8$; STZ + GW3965, $n = 6$. Statistical analysis was performed by one-way ANOVA followed by Tukey–Kramer posttest. *$p < 0.05$, **$p < 0.001$ versus control rats.

Table 3. Body weight and blood chemistry of control, STZ, and STZ rats treated with GW3965

	Control	STZ	STZ + GW3965
Weight (g)	524.1 ± 60.4	270.4 ± 46.4**	288.8 ± 50.4**
Glycemia (mg/dl)	84.2 ± 19.2	953.1 ± 238.1**	949.7 ± 207.9**
Triglycerides (mg/dl)	68.9 ± 19.2	83.6 ± 38.6	89.5 ± 47
NEFA (mEq/L)	0.70 ± 0.24	0.67 ± 0.31	0.72 ± 0.28

Data are expressed as the mean ± SEM, $n = 14$. Statistical analysis was performed by one-way ANOVA followed by Tukey–Kramer posttest. *$p < 0.05$, **$p < 0.001$ versus control rats. NEFA, nonesterified fatty acids.

LXR ligands reduce diabetes-induced neuropathy

Since activation of LXRs in diabetic sciatic nerves restored neuroactive steroid levels to a near-normal state, we examined whether this LXR-mediated increase could be associated with a neuroprotective effect. Table 3 shows that 3 months after the induction of diabetes, STZ-treated rats had higher blood glucose and significantly lower weight than nondiabetic control rats. Plasma levels of triglycerides and nonesterified fatty acids were unaffected by diabetes. Treatment with an LXR agonist had no effect on these parameters. However, LXR activation did result in significant neuroprotective effects as measured by functional and biochemical tests. Treatment with GW3965 was able to significantly reduce the increase in thermal sensitivity brought about by diabetes (Fig. 3). In addition to this decrease in thermal nociceptive threshold in LXR ligand-treated animals, we also observed that antidromic tail nerve conduction velocity (NCV), which is significantly reduced by diabetes, was enhanced by LXR ligand treatment. Moreover, treatment with the LXR agonist reverted the reduction in Na^+, K^+-ATPase activity in sciatic nerve brought about by diabetes (Fig. 3). In contrast to these neuroprotective effects mediated by LXR activation, treatment with GW3965 did not consistently counter the diabetes-induced decrease in expression of myelin proteins (i.e., P0, PMP22, MAL and MAG) observed in the sciatic nerve of STZ-treated animals, even if a tendency to reach significance was observed in case of P0 and PMP22 (Fig. 4).

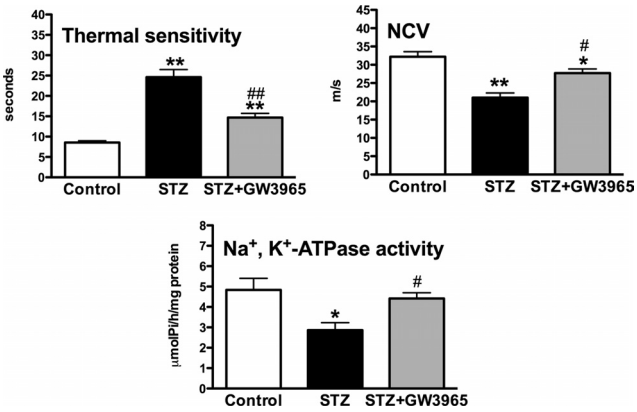

Figure 3. Thermal sensitivity, nerve conduction velocity, and Na^+, K^+-ATPase activity in control, STZ and STZ treated with GW3965 rats. Data are expressed as withdrawal latency in seconds for heat sensitivity threshold (control, $n = 10$, STZ, $n = 12$; STZ + GW3965, $n = 14$), as m/s for NCV (control, $n = 14$; STZ, $n = 12$; STZ + GW3965, $n = 14$) and as μmol Pi/h per mg protein for Na^+, K^+-ATPase (control, $n = 6$; STZ $n = 7$; STZ + GW3965, $n = 6$), and are mean ± SEM. Statistical analysis is performed by one-way ANOVA followed by Tukey–Kramer posttest. *$p < 0.05$, **$p < 0.001$ vs control rats; #$p < 0.05$, ##$p < 0.001$ vs STZ-treated rats.

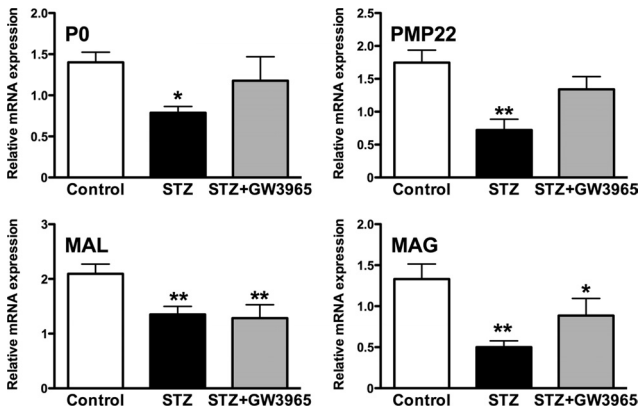

Figure 4. Gene expression of myelin proteins in sciatic nerve. The bars represent the relative mRNA expression of shown genes to the housekeeping gene 36B4. Data are presented as mean ± SEM ($n = 9$). Statistical analysis is performed by one-way ANOVA followed by Tukey–Kramer posttest. *$p < 0.05$, **$p < 0.001$ vs control rats.

Discussion

Diabetes is a leading cause of mortality worldwide. Chronic hyperglycemia usually results in complications such as high blood pressure, blindness, kidney malfunction, and nervous system disease (ADA, 2007; Herman, 2007). Diabetic neuropathy appears frequently in patients diagnosed with either type 1 or type 2 diabetes; in fact, 60% to 70% of diabetics develop nerve injuries. Nerve malfunction and damage is primarily due to decreased blood flow and high blood glucose levels; the extent of abnormalities is more pronounced if the hyperglycemia is not controlled properly. Peripheral nerve injuries may affect cranial nerves or nerves from the spinal column and their branches (Sugimoto et al., 2000; Vinik et al., 2000).

Neuroactive steroids have been shown to exert neuroprotective effects in experimental models of diabetic neuropathy (Leonelli et al., 2007). We have recently shown that increasing cholesterol shuttling into the mitochondria using a TSPO ligand resulted in an increase in the low levels of neuroactive steroids present in the sciatic nerve of diabetic animals that was accompanied by nerve protective effects (Giatti et al., 2009). Here, we report similar findings using synthetic activators of the LXR nuclear receptors. The low levels of PREG (the first steroid formed from cholesterol) measured in sciatic nerve of STZ-treated diabetic animals (Pesaresi et al., 2010) were increased upon LXR ligand treatment. LXR activation is known to play a role in steroidogenesis in the adrenal gland, directly regulating StAR expression, an important molecule involved in the initial process of steroidogenesis (Cummins and Mangelsdorf, 2006). In agreement with these results, we find that in peripheral nerves, such as the sciatic nerve, activation of LXR in the context of diabetes restores normal StAR mRNA levels. Moreover, we also find that the mRNA levels of P450scc, the enzyme converting cholesterol to PREG, are completely restored to nondiabetic levels in STZ-treated rats dosed with GW3965. In contrast, TSPO expression was not influenced by treatment with GW3965. Whether simultaneous activation of StAR and TSPO is necessary to activate steroidogenesis is a subject of debate (Bogan et al., 2007; Rone et al., 2009). In our study, the normalization of StAR and P450scc mRNA levels may be sufficient to account for the increased levels of PREG measured in sciatic nerve. We also found restored levels of PROG and its metabolite DHP, as well as metabolites of T, such as 3α-diol, in the sciatic nerve of diabetic rats treated with the LXR ligand. At least in the case of PROG and DHP, the increased levels observed in LXR-treated diabetic rats may be due to greater availability of their precursors (e.g., PREG), as well as to increased expression of the enzyme that generates DHP (e.g., 5α-reductase). Interestingly, LXR activation did not affect plasma levels of PREG, PROG, DHP and 3α-diol demonstrating that the increase on neuroactive steroids levels we observed was due to enhanced local production and not to uptake from the periphery. This observation is quite significant because increasing levels of neuroactive steroids directly in the nervous system and not in plasma, may avoid possible endocrine side effects exerted by these molecules. These findings contrast with what we previously observed on diabetic animals treated with a TSPO ligand. Treatment with Ro5-4864 led to an increase not only of PREG and PROG, as with GW3965 treatment, but also of DHT (a metabolite of T). Moreover, the increase of PROG and T levels occurred locally and in plasma (Giatti et al., 2009). These observations suggest somewhat different mechanisms of action for LXR and TSPO ligands, an issue to be explored in future experiments.

Because LXR activation restored local production of neuroactive steroids, we tested whether these effects resulted in neuroprotection in diabetic STZ-treated rats. Treatment with an LXR ligand ameliorated the impairment in NCV, thermal threshold, and Na^+, K^+-ATPase activity brought about by the diabetic state. It is interesting to note that these functional and biochemical parameters are also affected by TSPO ligand (Giatti et al., 2009). However, at variance to what we observed with TSPO ligand, treatment with LXR ligand did not significantly affect expression of myelin proteins, suggesting yet again that the mechanism of action by which LXR and TSPO activation evoke protective effects in peripheral nerve may be different. These results are in agreement with what we have observed in diabetic rats treated with PROG, T or their metabolites (Leonelli et al., 2007; Roglio et al., 2007). It is likely that LXR activation exerts its neuroprotective effects primarily by increasing the levels of PREG, PROG, DHP and 3α-diol in the sciatic nerve. In this context, it is important to remember that these neuroactive steroids wield their effects via classical and nonclassical steroid receptors (i.e., PROG and DHP are ligands of the progesterone receptor while 3α-diol activates the GABA-A receptor and estrogen receptor β) (Melcangi et al., 2008). Thus, a participation of these receptors in neuroprotective effects exerted by LXR ligand may be hypothesized. LXR activation may impact neuroactive steroid levels through its ability to regulate transcription of genes involved in cholesterol homeostasis. In this study, expression of HMGCoA reductase and SREBP-2, key regulators of cholesterol synthesis, was unchanged among experimental groups, suggesting that enhanced cholesterol synthesis is not responsible for the increased neuroactive steroid levels seen with LXR activation in the sciatic nerve. Thus, we hypothesize that the benefits of LXR activation in this setting are due to a promotion of cholesterol utilization, similar to what is seen in the adrenal gland (Cummins et al., 2006). In support of this idea, we observed an upregulation of cholesterol efflux genes, such as ABCA1 and ABCG1, in the sciatic nerve of animals treated with GW3965. These genes are direct LXR targets, and their induction would be expected to maintain the level of free cholesterol at a safe limit by promoting cholesterol efflux.

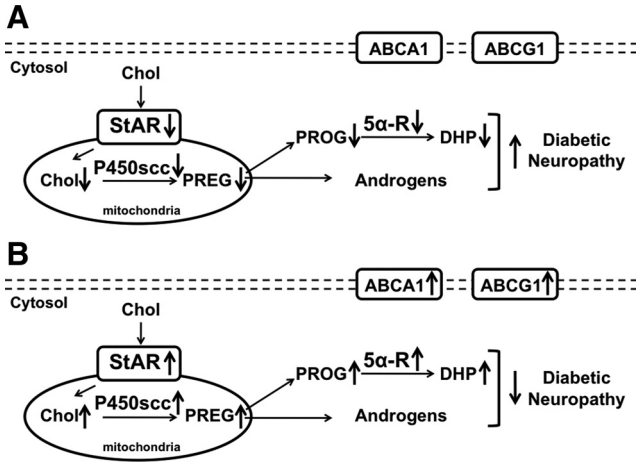

Figure 5. Proposed model of LXRs activation in diabetic neuropathy. The entrance of cholesterol into mitochondria is accomplished by the steroidogenic acute regulatory protein (StAR), a transport protein that regulates cholesterol transfer from the outer mitochondrial membrane to the inner membrane. Here, cholesterol is the substrate of P450scc enzyme, the first enzymatic step in the neuroactive steroid synthesis. ***A***, In the diabetic state, we observed a reduced neuroactive steroid synthesis in the sciatic nerve due to decreased expression of StAR, P450scc and 5α-reductase (5α-R). ***B***, The treatment of diabetic rats with the GW3965, a LXR synthetic ligand, restored the expression of the steroidogenic enzymes, and the neuroactive steroid levels affected by diabetic neuropathy. Moreover, LXR activation also induced the expression of the cholesterol efflux genes such as ABCA1 and ABCG1. In conclusion, the activation of LXRs promotes cholesterol utilization and finally protects from peripheral neuropathy-induced diabetes.

In summary, as shown in Figure 5, we demonstrate that LXR activation in a diabetic setting that results in peripheral neuropathy can have a beneficial effect. LXR activation promotes steroidogenesis, cholesterol disposal, and raises the local levels of neuroactive steroids. These effects are associated with neuroprotection against peripheral neuropathy induced by diabetes. The present data extend the neuroprotective actions of LXR so far observed in multiple neuronal injury models, such as ischemic brain injury, Alzheimer's disease, and Niemann-Pick C disease (Repa et al., 2007; Zelcer et al., 2007; Morales et al., 2008; Sironi et al., 2008; Cheng et al., 2010). Finally, we are suggesting for the

first time that LXR may be a promising therapeutic target for diabetic neuropathy.

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