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Rimonabant, a cannabinoid CB_1 receptor antagonist, attenuates mechanical allodynia and counteracts oxidative stress and nerve growth factor deficit in diabetic mice

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ABSTRACT

Diabetes is one of the leading causes of painful neuropathy and to date, besides a tight glycemic control, a viable treatment for this complication is not available. Rimonabant is a selective cannabinoid CB_1 receptor antagonist that produces a significant increase in insulin sensitivity and a reduction of HbA_{1c} in diabetic patients. This study aimed to investigate the therapeutic potential of rimonabant in relieving diabetes-induced neuropathic pain. The repeated treatment with rimonabant evoked a significant attenuation of mechanical allodynia in diabetic mice that was dose- and time-dependent. This effect occurred without alteration of hyperglycemia, but it was associated with significant effects on many key players in the pathogenesis of diabetic neuropathy. Metabolic changes induced by hyperglycemia lead to oxidative stress, deregulation of cytokine control and reduced production and transport of nerve growth factor (NGF), and all these factors contribute to neuropathic pain. Rimonabant treatment reduced oxidative stress in peripheral nerve, as revealed by the ability of the compound to counteract the reduced glutathione (GSH) depletion. The same repeated treatment inhibited tumor necrosis factor (TNFα) overproduction in the spinal cord and increased the NGF support. This rimonabant-induced improvement might favour the nerve regeneration; accordingly, the histological analysis of sciatic nerves showed a marked degeneration of myelinated fibers in diabetic mice, that was substantially reduced after rimonabant administration. These findings support the hypothesis that CB_1 antagonists would represent a new opportunity for diabetic patients, since currently there are no treatments for painful diabetic neuropathy other than treating the diabetic condition per se.

1. Introduction

Neuropathy is the most common complication of diabetes, occurring in about 60% of all diabetic patients (Feldman et al., 1999). Diabetic neuropathy is characterized by a slowly progressive loss of sensation that correlates with diabetic duration and glycemic control. Pharmacological treatment of neuropathic pain is fundamentally different from the treatment of nociceptive pain, with regular analgesics only partially effective. In respect to diabetic neuropathy, to date, besides a tight glycemic control, a viable treatment for the human disease is not available. The diabetic neuropathy is a significant cause of morbidity and mortality since the epidemic explosion of diabetes throughout the world and since it is among the most common long-term complications of diabetes. Thus, despite progress in analgesic drug discovery, the need for therapeutic agents capable of blocking abnormal pain sensation without impairing normal abilities remains largely unmet. Among the various emerging therapeutic new substances, cannabinoids are promising analgesics (Lever and Rice, 2007 for review). Cannabinoids are lipophilic compounds originally obtained from *Cannabis sativa* which contains more than 60 different cannabinoids. Phytocannabinoids obtained from the cannabis plant comprise a range of cannabinoid receptor agonists, partial agonists, and antagonists. There are two main cannabinoid receptors, CB_1 and CB_2, associated with pain modulation. The first is widely expressed in the CNS and in peripheral sensory neurons, whereas the latter has been found in peripheral tissues, including tissues of the immune system and keratinocytes, with limited expression in sensory and CNS cells (Pertwee, 2006 for review). Endogenous cannabinoids have been also well characterized (i.e. anandamide and 2-arachidonoylglicerol). Of interest, the endocannabinoid anandamide can also activate the transient receptor potential vanilloid 1 (TRPV1) channel, suggesting that anandamide also behaves as endovanilloid (Zygmunt et al., 1999; Smart et al., 2000). TRPV1 receptor is a molecular sensor of noxious heat and chemicals (capsaicin), but it can also be modulated by low pH and lipids and it is highly expressed on primary sensory neurons (Caterina and Julius, 2001). Both TRPV1 antagonists, through the blockade of the receptor, and agonists, through the desensitization of the receptor, behave as analgesics.

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Many synthetic cannabinoids have also been developed with specific receptor affinity and distinct pharmacological profiles. Rimonabant is a selective cannabinoid CB_1 receptor antagonist that has recently undergone testing in the treatment of obesity in human subjects. Particularly, the Rimonabant in Obesity Diabetes (RIO-Diabetes) trial enrolling overweight and obese patients on monotherapy for treatment of type 2 diabetes (Scheen et al., 2006) showed that the drug produced a significant reduction in the glycosylated hemoglobin (HbA_{1c}) so leading to an improvement of the glycaemic equilibrium that seems crucial in preventing and treating the late complications of diabetes, including neuropathy. In addition, we recently demonstrated that the repeated oral administration of rimonabant dose-dependently attenuated both thermal and mechanical hyperalgesia in a murine model of neuropathic pain due to nerve injury (chronic constriction injury of sciatic nerve model) (Costa et al., 2005). On these bases this study aimed to investigate the therapeutic potential of the repeated treatment with rimonabant in relieving neuropathic pain in a widely used animal model of diabetic neuropathy, that is the streptozotocin-induced diabetes in mice.

2. Material and methods

2.1. Animals

All experiments performed were in accordance with Italian and European regulations governing the care and treatment of laboratory animals (Permission no. 41/2007B) and conformed to the guidelines for the study of pain in awake animals established by the International Association for the Study of Pain (Zimmermann, 1983). All efforts were made to minimize the number of animals used and their discomfort. Male C57BL6/J mice weighing 25–30 g (Harlan, Italy) were housed under controlled temperature (22 ± 1 °C), humidity ($60 \pm 10\%$) and light (12 h/day) and allowed to acclimatise for at least one week before use in the experiments. Standard food and water were available *ad libitum*.

2.2. Induction of diabetes

Type 1 diabetes was induced through chemical pancreatectomy by a single intraperitoneal (i.p.) injection of streptozotocin (Sigma, Italy) at 120 mg/kg, freshly prepared in citrate buffer 0.1 M pH 4.5. We have previously shown that rimonabant, repeatedly administered to naive animals did not affect pain responses in mice, but it decreased thermal latency in rats (Costa et al., 2005). Accordingly, while some studies showed an intrinsic hyperalgesic effect of rimonabant in rats (Chapman, 1999; Herzberg et al., 1997; Strangman et al., 1998), other studies failed to find such activity in mice (Rinaldi-Carmona et al., 1994; Welch et al., 1998). This important issue, together with the established fact that, as for other pain models originally developed in rats, the streptozotocin-induced diabetic neuropathy has been successfully transposed in mice (Colleoni and Sacerdote, in press for review), prompted us to employ mice rather than rats in the present study. Diabetes was verified one week later by measurement of blood glucose concentration by a glucometer (Lifescan One Touch Ultra glucose meter, Milan, Italy) on a sample of blood obtained from a tail prick. Only mice with blood glucose levels above 250 mg/dl were selected for the experiments. Control mice received an i.p. injection of citrate buffer. Blood glucose level and mice body weight were monitored over the whole period of the experimental study.

2.3. Drugs and treatments

Rimonabant was dissolved in a 1:2:7 mixture of Tween80:DMSO: distilled water and used at the doses of 3 and 5 mg/kg. Capsazepine (Sigma, Italy) was dissolved in 10% DMSO and 90% saline and employed at 5 mg/kg. Diabetic mice, randomly divided, received intraperitoneally (100 µl/10 g body weight) the rimonabant or its vehicle, once a day for 7 days, starting from the 14th day after the diabetes induction. Control mice received drug vehicle. For the antagonism study diabetic mice, randomly divided into four groups, received rimonabant alone (5 mg/kg, 50 µl/10 g body weight), or the selective TRPV1 receptor antagonist, capsazepine alone (5 mg/kg, 100 µl/10 g body weight) or the two compounds (capsazepine 5 min before rimonabant) or the drug vehicles. The i.p. administrations were performed daily for one week starting from the 14th day after streptozotocin.

2.4. Mechanical allodynia

Responses to non noxious mechanical stimulus were measured before diabetes induction, on day 14 (before starting the treatment) and on days 18 and 21 (24 h after the last administration of the compound). Mechanical allodynia was assessed using the Dynamic Plantar Aesthesiometer (Ugo Basile, Varese, Italy). Animals were placed in a test cage with a wire mesh floor, and allowed to acclimate for 30 min. The tip of a von Frey-type filament was applied to the middle of the plantar surface of the hind paw. Gradually increased pressure was applied and the stimulus was continued until the hind paw was withdrawn or slowly elevated (actions such as vocalization, agitation, jumping, and avoidance were considered indicative of the withdrawal threshold, too). The force required to elicit a withdrawal response was recorded twice for each hind paw with an interval of at least 1 min and averaged. Withdrawal threshold was expressed as threshold level in g.

2.5. Biochemical evaluations

Twenty-one days following streptozotocin injection, 24 h after the last administration of rimonabant or its vehicle, pain assessment was recorded and animals were sacrificed. Sciatic nerves proximal to the trifurcation were quickly removed; part of them was immediately stored at −80 °C until the neurotrophic factor nerve growth factor (NGF) was assayed and part was processed to obtain the cytosolic fraction for the reduced glutathione (GSH) determination, as described afterwards. A set of sciatic nerves was harvested and processed to evaluate axon morphology. Livers were removed. Part of them was processed to obtain cytosolic fraction for the GSH assay. Briefly, livers and sciatic nerves were washed with ice-cold saline solution, homogenized in four volumes of ice-cold 0.15 M KCl and centrifuged at $9000 \times g$, at 4 °C for 10 min. Supernatants were successively ultra centrifuged at $100{,}000 \times g$, at 4 °C for 1 h in order to obtain cytosolic fraction which was stored at −80 °C until analyzed. Part of liver was stored at −20° and used for malondialdehyde (MDA) assay. The spinal cord was removed, frozen in liquid nitrogen, and stored at −80 °C until the determination of tumor necrosis factor (TNFα) content.

2.5.1. GSH assay

The GSH content was determined fluorimetrically with 350 nm and 420 nm as excitation and emission wavelengths, according to the method of Hissin and Hilf (1976), using ophthalaldehyde (OPT) as fluorescent reagent. The GSH concentration was calculated using a standard curve with known amounts of GSH and is expressed as µg GSH/mg protein for liver and µmol GSH/g tissue for sciatic nerve.

2.5.2. MDA assay

MDA level, an indicator of free-radical generation, was estimated by the thiobarbituric acid (TBA) test of Ohkawa et al. (1979). Briefly, livers were homogenated with potassium phosphate (50 mM)–EDTA (0.1 mM) buffer, pH 7.4 (1:10, w:v). The concentration of MDA was established spectrophotometrically at 532 nm, calculated employing the extinction coefficient of the MDA–TBA complex ($0.156\ \mu M^{-1}\ cm^{-1}$) and expressed as nmol MDA/g wet weight tissue.

2.5.3. NGF assay

Sciatic nerves were homogenized in a cold lysis buffer (200 µl). The homogenates were centrifuged at 4500×g at 4 °C for 10 min, and the resulting supernatants were then diluted 5-fold with Dulbecco's PBS buffer. Samples were acidified to pH<3.0 by adding 1 N HCl, briefly mixed and then neutralized with 1 N NaOH to pH 7.6. Samples were then centrifuged 10,000×g at 4 °C for 15 min and the resulting supernatants used to determine NGF protein levels by enzyme-linked immunosorbent assay (ELISA) using ELISA kit according to the manufacturer's instructions (Promega, USA). The absorbance at 450 nm was recorded on a microplate reader (Multiskan® EX, ThermolabSystem). NGF levels were determined by interpolation with standard curves assayed on individual plates, normalized to protein content in each tissue sample and expressed as pg NGF/mg protein.

2.5.4. TNFα assay

The determination of TNFα protein levels was performed using an enzyme-linked immunosorbent assay (ELISA) in the spinal cord. Spinal cord tissue was weighed and homogenized in phosphate-buffered saline (PBS), pH 7.4, containing a mix of protease inhibitors, in a ratio of 10 ml of PBS/mg spinal cord. After centrifugation at 10,000×g at 4 °C for 10 min, the supernatant was removed and assayed in duplicate by the mouse TNFα Ultrasensitive ELISA kit (Biosource International, Camarillo, CA, USA) according to the manufacturer's instruction. Data are expressed as pg TNFα/mg protein.

2.6. Histology of sciatic nerves

An approximately 8 mm-long segment of the sciatic nerve (left and right from each mouse) was removed and immediately fixed in modified Karnovsky's liquid (2% paraformaldehyde and 0.2% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4), overnight. Tissue was repeatedly rinsed with phosphate buffer and post-fixed in 1% osmium tetroxide for 1 h, dehydrated in serial alcohol concentrations and embedded in Epon resin. Transversal semithin sections were cut on a LKB ultramicrotome. About 100 sections were taken for each animal, stained with 0.2% toluidine blue and examined under a light Zeiss Axioplan MC 100 microscope. Thirty sections randomly selected were quantified and averaged from each animal. Images were taken by a colour digital camera (Image ProPlus version 4.5.1, Media Cybernetics). After acquisition of section images, intact myelinated fibers were counted by an experimenter blinded to the pharmacological treatment and expressed as density (number per square millimeter).

2.7. Statistics

All data are expressed as the mean ± S.E.M. and analyzed using one-way ANOVA followed by Tukey's post-hoc test. Differences were considered significant at $P<0.05$.

3. Results

3.1. Effect of rimonabant on diabetes-induced mechanical allodynia

As expected, streptozotocin-injected mice developed pain-related behaviour, marked as mechanical allodynia. Particularly, two weeks after streptozotocin administration, significant reductions in paw withdrawal thresholds to von Frey filament were observed (Fig. 1). The mechanical allodynia was still present in vehicle-treated animals during the subsequent week (3.12 g ± 0.08 vs 4.89 g ± 0.05 for non-diabetic mice), whereas mice systemically treated with rimonabant once a day for one week displayed a significant time- and dose-related attenuation of mechanical hypersensitivity (Fig. 1) (3.78 g ± 0.06 and 4.54 g ± 0.08 for 3 mg/kg and 5 mg/kg, respectively). The highest dose of rimonabant (5 mg/kg) led to a response to the von Frey stimulus similar to that of control (non-diabetic) mice (Fig. 1). The same dose

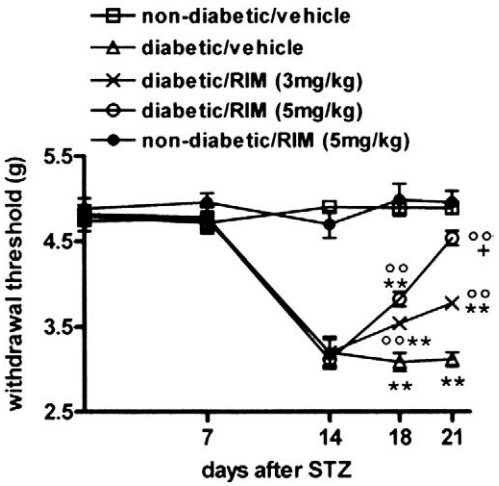

Fig. 1. Effect of rimonabant (RIM) i.p administered daily to diabetic mice for one week, starting from day 14 after streptozotocin injection, on mechanical allodynia. The effect of the highest dose of rimonabant repeatedly i.p. administered to control (non-diabetic) mice is also shown. Data represent mean ± S.E.M. of 6–10 mice. $^{**}P<0.01$ vs non-diabetic/vehicle; $^{\circ\circ}P<0.01$ vs diabetic/vehicle; $^{+}P<0.05$ vs diabetic/RIM (3 mg/kg) by one-way ANOVA followed by Tukey's test.

of compound did not affect the mechanical perception in non-diabetic mice (4.96 g ± 0.13) (Fig. 1).

3.2. Effect of co-administration of rimonabant and capsazepine on diabetes-induced mechanical allodynia

To ascertain whether TRPV1 receptor played a role in the rimonabant-induced relief of mechanical allodynia in diabetic mice, animals were co-administered with a TRPV1 antagonist, capsazepine (5 mg/kg, i.p.), and rimonabant (5 mg/kg, i.p.) once a day for one week, starting the 14th after streptozotocin injection. The withdrawal thresholds to von Frey-type filament have been evaluated 24 h after the last administration. The TRPV1 receptor antagonist, capsazepine, when administered alone to diabetic mice, did not affect their nociceptive response (3.13 g ± 0.15 vs 3.24 g ± 0.09 for diabetic/vehicle mice) (Fig. 2). However, capsazepine given with rimonabant reversed the antiallodynic action of rimonabant (2.98 g ± 0.08 for the combined treatment vs 4.74 g ± 0.13 for rimonabant alone) (Fig. 2).

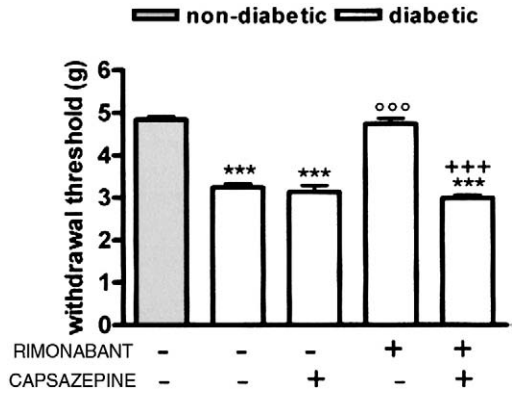

Fig. 2. Effect of daily co-administration of capsazepine (5 mg/kg, i.p.) and rimonabant (5 mg/kg, i.p.) to diabetic mice for one week, starting from day 14 after streptozotocin injection, on mechanical allodynia. Data represent mean ± S.E.M. of 5 mice. $^{***}P<0.001$ vs non-diabetic; $^{\circ\circ\circ}P<0.001$ vs diabetic; $^{+++}P<0.001$ vs diabetic/rimonabant by one-way ANOVA followed by Tukey's test.

Table 1
Metabolic parameters in diabetic mice treated with vehicle or rimonabant (5 mg/kg, i.p.) for one week starting from day 14 after streptozotocin, as compared to control (non-diabetic) mice.

Parameter (unit)	Non-diabetic	Diabetic	Diabetic/rimonabant
Blood glucose (mg/dl)	145.8 ± 9.024	530.3 ± 58.08[a]	554.0 ± 27.78[a]
Body weight gain (g)	3.00 ± 0.408	−3.33 ± 0.333[a]	−1.00 ± 0.577[a,b]
Hepatic GSH (μg/mg protein)	20.74 ± 0.720	14.54 ± 1.352[a]	14.93 ± 1.614[a]
Hepatic MDA (nmol/g tissue)	142.3 ± 19.05	319.3 ± 14.94[a]	263.3 ± 19.39[a]

Values are mean ± S.E.M. of 6–10 mice.
[a] $P<0.05$ vs non-diabetic.
[b] $P<0.05$ vs diabetic by one-way ANOVA followed by Tukey's test.

3.3. Effect of rimonabant on diabetes-induced hyperglycemia and body weight loss

By the 7th day after administration of streptozotocin, mice developed hyperglycemia: their blood glucose level (399.7 ± 51.10 mg/dl) was statistically different from that of control animals (155.5 ± 3.10 mg/dl). Hyperglycemia became greater at week two (490.1 ± 42.06 mg/dl for diabetic mice vs 145.3 ± 11.63 mg/dl for non-diabetic mice). The body weight of diabetic mice became significantly lower than that of controls by the first week after streptozotocin with a decrease of about 1.5 g of body weight vs an increase of about 1.5 g of non-diabetic mice after 14 days from streptozotocin. Starting from this time point, diabetic mice were daily treated with vehicle or rimonabant (5 mg/kg). As shown in Table 1, the blood glucose level of mice treated for one week with rimonabant did not differ from that of diabetic mice treated with vehicle, showing that the pharmacological treatment did not modify the hyperglicemia induced by streptozotocin. Conversely, the repeated administration with rimonabant evoked an attenuation of the body weight loss of diabetic mice. Particularly, Table 1 shows that the decrease in body weight gain of vehicle-treated diabetic mice is more of 3 g, whereas that of rimonabant-treated diabetic mice is only of 1 g, probably as a consequence of the pain reduction with an amelioration of the general condition of mice. Generally, diabetic animals are chronically ill and, besides body weight loss, displayed poor body conditions: polyuria, increased water intake, dehydration, muscle wasting, and diarrhea. In addition, general activity is greatly reduced and the animals become extremely lethargic, to the point where their exploratory behaviour is reduced. In our hands the most severe decline of general condition developed within the first week after streptozotocin, when there was also a 5% of mortality rate. Afterwards, animals stabilized their general condition to an acceptable level, whereas those showing a very poor general condition were removed from the study. In such a scenario mice repeatedly given rimonabant did not show any substantial changes in overt behaviour as compared to control diabetic mice, even if their general activity increased, probably as a consequence to the pain relief.

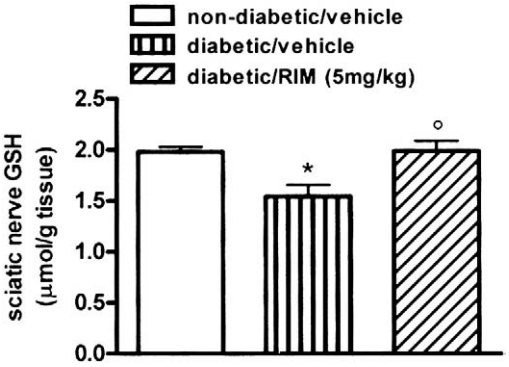

Fig. 3. Effect of rimonabant (RIM) i.p administered daily to diabetic mice for one week, starting from day 14 after streptozotocin injection, on GSH level in the sciatic nerve. Data represent mean ± S.E.M. of 5 mice. *$P<0.05$ vs non-diabetic/vehicle; °$P<0.05$ vs diabetic/vehicle by one-way ANOVA followed by Tukey's test.

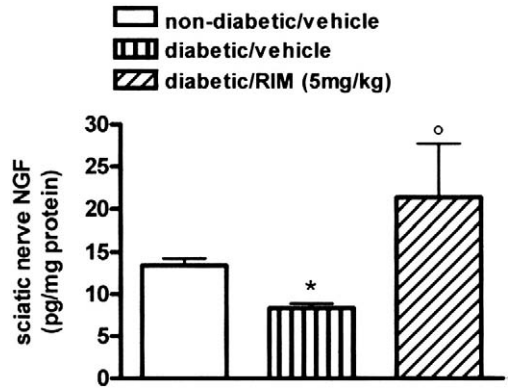

Fig. 4. Effect of rimonabant (RIM) i.p administered daily to diabetic mice for one week, starting from day 14 after streptozotocin injection, on NGF level in the sciatic nerve. Data represent mean ± S.E.M. of 5 mice. *$P<0.05$ vs non-diabetic/vehicle; °$P<0.05$ vs diabetic/vehicle by one-way ANOVA followed by Tukey's test.

3.4. Effect of rimonabant on diabetes-induced oxidative stress

Diabetes-induced oxidative stress was evidenced as a GSH depletion (30%) and lipoperoxide increase (125%) in the liver of diabetic mice (Table 1). The repeated treatment with rimonabant did not affect the strong reduction of GSH and evoked only a slight, but not significant, reduction in the level of membrane lipoperoxidation (Table 1), indicating that the drug was not able to counteract the oxidative injury in the liver of diabetic mice. However, when the evaluation of the antioxidant peptide was performed in the sciatic nerve, it was possible to show that nerve GSH content (μmol/g tissue) was decreased by 22% in diabetic animals (1.54 ± 0.11) vs controls (1.98 ± 0.05), and that this decrease was essentially corrected in the nerve of mice repeatedly treated with rimonabant (1.99 ± 0.10) (Fig. 3).

3.5. Effect of rimonabant on NGF level in the sciatic nerve

Diabetes induced a significant reduction (38%) of NGF level (pg/mg protein) in the sciatic nerve of mice treated with vehicle for one week (8.34 ± 0.54 vs 13.41 ± 0.82) (Fig. 4). The repeated treatment with rimonabant elicited a strong increase in the NGF content at the level of sciatic nerve that was in fact three times higher than that found in diabetic mice (21.34 ± 6.34) (Fig. 4).

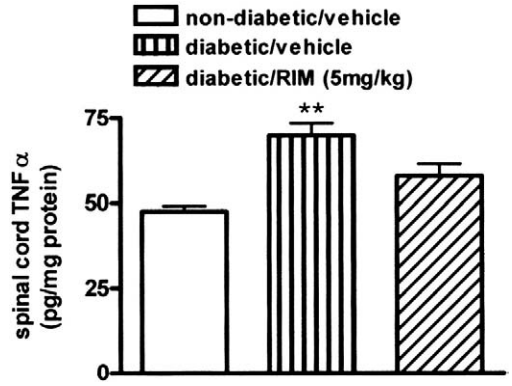

Fig. 5. Effect of rimonabant (RIM) i.p administered daily to diabetic mice for one week, starting from day 14 after streptozotocin injection, on TNFα in the spinal cord. Data represent mean ± S.E.M. of 6–10 mice. **$P<0.01$ vs non-diabetic/vehicle by one-way ANOVA followed by Tukey's test.

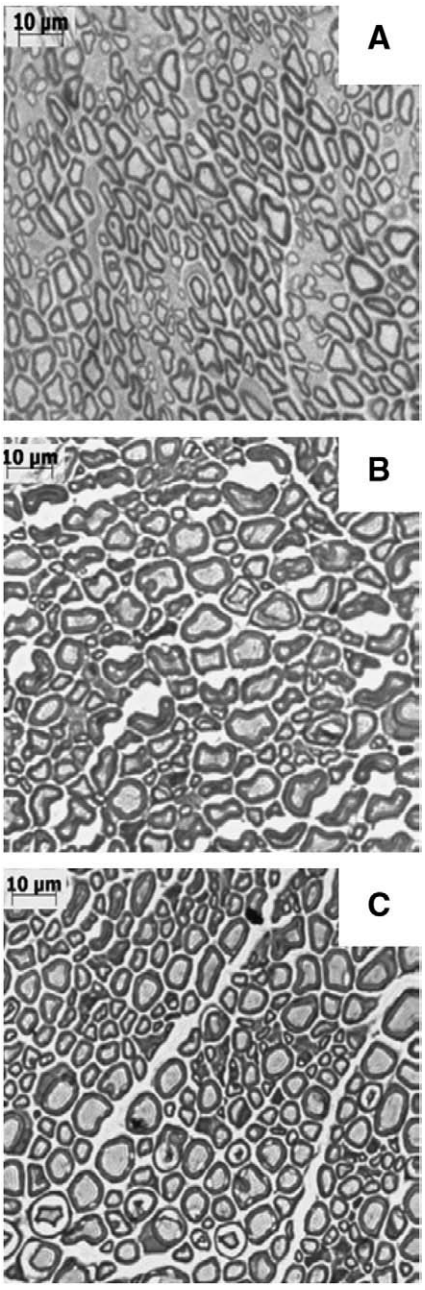

Fig. 6. Transverse semithin section of sciatic nerve from control (non-diabetic) mouse (A) and from diabetic mouse treated with vehicle (B) or with rimonabant (5 mg/kg, i.p.) (C) once a day for one week starting from day 14 after streptozotocin injection.

3.6. Effect of rimonabant on TNFα level in the spinal cord

Determination of TNFα by ELISA revealed higher levels (47%) in the spinal cord of diabetic mice (69.86 ± 3.61 pg/mg protein) when compared to non-diabetic animals (47.50 ± 1.57 pg/mg protein) (Fig. 5). Repeated administration of rimonabant resulted in a significant reduction in the level of TNFα (57.87 ± 3.52 pg/mg protein) as compared to diabetic mice treated with vehicle, even if the treatment did not restore the physiological level of this cytokine (Fig. 5).

3.7. Effect of rimonabant on nerve demyelination

The histological analysis performed on semithin sections of sciatic nerves stained with toluidine blue highlighted an extensive demyelination, myelin abnormalities, splitting of myelin lamellae, phagocytic Scwhann cells, and disorganization of the extracellular matrix at both 14 (not shown) and 21 days after streptozotocin injection (Fig. 6B), as compared to non-diabetic mice (Fig. 6A). Diabetic mice treated with rimonabant (5 mg/kg) from day 14 to day 21 showed evidence of nerve regeneration (Fig. 6C), with the presence of regenerating clusters and small remyelinated fibers. The average density of normal nerve fibers from each group is shown in Fig. 7. A significant reduction of myelinated fibers was shown in diabetic mice treated with vehicle (2803 ± 483.1) as compared to control animals (10,444 ± 321.3). Such a reduction was significantly lesser in the sciatic nerves of diabetic mice treated with rimonabant (5235 ± 922.9), even if the average density was still significantly different from that of control mice.

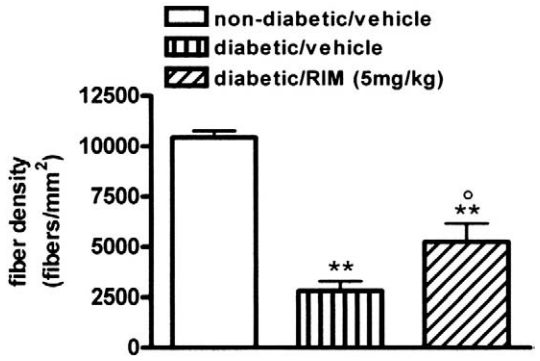

Fig. 7. Effect of rimonabant (RIM) i.p administered daily to diabetic mice for one week, starting from day 14 after streptozotocin injection, on fiber density in the sciatic nerve. The evaluation of intact fibers has been performed as described in the Material and methods section. Data represent mean ± S.E.M. of 5 mice. $^{**}P<0.01$ vs non-diabetic/vehicle; $^{\circ}P<0.05$ vs diabetic/vehicle by one-way ANOVA followed by Tukey's test.

4. Discussion

Diabetes is one of the leading causes of painful diabetic neuropathy, a disorder characterized by damage to peripheral nerves resulting in primary afferent nociceptors hyperexcitability (peripheral sensitization) that leads to hyperexcitability in central neurons (central sensitization). Sensitization is characterized by a lowered activation threshold, increased response to a given stimulus, and abnormal spontaneous activity (Baron, 2000). Treatment options for diabetic neuropathy are limited and it has been estimated that these therapies led at best to 50% reduction of pain in 50% of patients. Glycaemic control is important to prevent progression of neuropathy, and intensive glucose lowering therapy reduces the risk of developing diabetic neuropathy (Martin et al., 2006), suggesting that the prevention of diabetic neuropathy remains the best strategy. However, once pain develops, current treatments are not specific for the underlying cause of nerve damage; the future goal in treating diabetic neuropathy should be not only to prevent or delay the painful neuropathic symptoms, but also to completely relieve pain and possibly to promote the regeneration of degenerate nerve fibers after the onset of the pathology. Rimonabant is a selective cannabinoid CB_1 receptor antagonist that has been shown to improve insulin resistance and the profile of several metabolic and cardiovascular risk factors in diabetic obese patients (Scheen et al., 2006). Furthermore, rimonabant treatment is associated with improvements in HbA_{1c} with a greater effect in patients with more severe hyperglycaemia at baseline (Rosenstock et al., 2008). In the clinical trials mentioned above, effects associated with pain were not reported, but the ability of rimonabant to ameliorate glycaemic equilibrium together with our previous findings demonstrating that rimonabant induced pain relief, diminished neuroinflammation and promoted remyelination of the damaged sciatic nerve in the chronic constriction injury model of

neuropathy (Costa et al., 2005), prompted us to investigate the possible antinociceptive action of rimonabant in diabetic neuropathy.

4.1. Repeated treatment with rimonabant alleviates diabetes-induced mechanical allodynia

Like peripheral nerve injury model, diabetic neuropathy is characterized by spontaneous pain, allodynia and alteration in thermal perception. These behavioural signs, which are analogous to human conditions of diabetic neuropathic pain, have been shown to develop in a widely employed animal model: streptozotocin-induced diabetic neuropathy (Courteix et al., 1993). Here we demonstrate that the repeated treatment with rimonabant evoked a significant attenuation of mechanical allodynia in diabetic mice. In fact, the repeated systemic administration of the compound produced increases in paw withdrawal thresholds, compared to corresponding values in the vehicle control group. This effect is dose-dependent and was elicited by doses of rimonabant, which did not markedly alter the nociceptive response in non-pathological control animals. The antiallodynic effect of rimonabant was also time-dependent; in fact, the compound significantly reduced diabetic neuropathy already after three administrations, but one week of treatment was necessary to obtain a full reversal of pain hypersensitivity. Moreover, the single administration of rimonabant did not affect the nociceptive thresholds of diabetic animals (data not shown). Concerning the mechanism of action, multiple hypotheses can be postulated. Since rimonabant acts as antagonist/inverse agonist at CB_1 receptor, an involvement of such a receptor is likely. Recent findings clearly demonstrated that the inhibition of CB_1 function, either through genetic deletion or inverse agonism, resulted in a diminished sensitization of TRPV1 receptor (Fioravanti et al., 2008). In addition, both receptors are expressed in dorsal root ganglia neurons (Ahluwalia et al., 2000), suggesting a functional crosstalk. Furthermore, in diabetic subjects small fiber (the unmyelinated C and the thinly myelinated Aδ) damage is responsible for the allodynia and both types of fibers constitutively express TRPV1. On these bases, it is possible to speculate that the suppression of the CB_1 receptor constitutive activity exerted by rimonabant leads to decreased TRPV1 sensitivity to stimuli with a consequent antiallodynic effect. The ability of a TRPV1 receptor antagonist to reverse the antiallodynic effect of rimonabant when co-administered during the whole period of treatment, strongly supports this hypothesis, suggesting that the constitutive activity of CB_1 receptor is required to maintain the functionality of TRPV1 receptor and that the blockade of CB_1 receptor by rimonabant indirectly modulates TRPV1 function leading to its desensitization. The administration of TRPV1 antagonist preserves the receptor from the desensitization evoked by CB_1 blockade, with consequent allodynia even following rimonabant treatment. This finding highlights the possibility of a modulation of TRPV1 function through means other than compounds with direct interaction at the channel. In the experiments performed herein, capsazepine was selected as TRPV1 antagonist, since it is able to inhibit agonist binding/activation of the channel, thus avoiding its desensitization, but it was ineffective if employed in mouse and rat models of neuropathic pain (Walker et al., 2003). In agreement, also in our hands the repeated administration of capsazepine alone was unable to counteract diabetes-induced allodynia. More recently, new TRPV1 antagonists have emerged, representing a new generation of compounds that overcome many of the hurdles plaguing the old TRPV1 antagonists, including capsazepine. These new antagonists have been found effective against chronic pain (see Szallasi et al., 2007 for review). However, when tested in clinical trials, the TRPV1 direct antagonists showed some unwanted effects, resulting in a less favourable risk/benefit profile than it was initially supposed (Gavva et al., 2008). Our findings highlighted the possibility to indirectly modulate this receptor and, although the full beneficial effect of such an indirect modulation of TRPV1 activity warrants further exploration, this approach might be really advantageous, since it should be possible to affect the TRPV1 function only where the two pathways (cannabinoid and vanilloid) coexist, avoiding the general TRPV1 receptor blockade responsible for the side effects of direct TRPV1 antagonists.

4.2. Rimonabant does not reduce hyperglycemia

We tested whether the rimonabant-induced relief of diabetic neuropathy can be due to an action upon hyperglycemia, since the latter has been reported to contribute to the development of diabetic neuropathy. However, the results showed that the repeated treatment with rimonabant did not result in a decrease of blood glucose level. This finding is in agreement with previous data obtained in diet-induced obese (DIO) rats showing no changes in plasma glucose level after i.p. administration of rimonabant (10 mg/kg/day for 6 days) (Nogueiras et al., 2008). Accordingly to our results, a clinical study reported a slight reduction of fasting glucose only following the highest dose of rimonabant (20 mg/day) and only after two years of treatment (Van Gaal et al., 2008).

4.3. Rimonabant counteracts GSH depletion in the sciatic nerve

One of the important consequences of chronic hyperglycaemia is enhanced oxidative stress resulting from imbalance between production and neutralization of reactive oxygen species. The membrane lipid peroxidation and the depletion of the key biological antioxidant, GSH, are the most relevant signs of the diabetes-induced oxidative stress (Vincent et al., 2004 for review). Our findings demonstrate that the repeated treatment with the CB_1 antagonist did not significantly affect the oxidative damage in the liver of diabetic mice, whereas it was able to counteract the GSH depletion in the sciatic nerve. The decrease in the sciatic nerve GSH level in diabetic mice in our study is consistent with previous reports (Bravenboer et al., 1992; Obrosova et al., 2001). In addition free-radical scavengers, such as N-acetylcysteine (a precursor of GSH synthesis) and pentoxifylline have been shown to inhibit the development of peripheral neuropathy in streptozotocin-induced diabetic rats without affecting the blood glucose level (Sagara et al., 1996; Qiang et al., 1998).

4.4. Rimonabant inhibits TNFα overproduction in the spinal cord

Emerging evidence highlight the role of TNFα inhibition in the relief of diabetic neuropathy; in fact, both N-acetylcysteine and pentoxifylline act as TNFα inhibitors and a study examined the effect of troglitazone, an anti-diabetic thiazolidinedione, on diabetic neuropathy, since it also is a free-radical scavenger and a TNFα inhibitor showing that it has a beneficial effect on peripheral neuropathy in streptozotocin-induced diabetic rats irrespective of blood glucose concentrations (Qiang et al., 1998). On this basis and in the light of our previous finding demonstrating that the effect of rimonabant on pain-related behaviour in chronic constriction injury model of neuropathy could be mediated by a down regulation of TNFα production in the spinal cord (Costa et al., 2005), we tested the consequence of rimonabant treatment on TNFα level in diabetic mice. Until now most studies demonstrated an enhancement of this cytokine in serum or plasma of patients (Gordin et al., 2008) or animals (Tanaka et al., 1992) during diabetic condition. Here we show that there is a significant increase in TNFα production in the spinal cord of diabetic mice, and this finding adds new insight concerning the fact that TNFα may play a pathogenic role in the development of diabetic neuropathy. Even if, basing on the data presented herein, we cannot rule out the possibility that cytokine dysregulation might be a consequence rather than a cause of neuropathic pain, previous study suggested that metabolic changes induced by hyperglycemia lead to dysregulation of cytokine control and that its regulatory roles in nerve degeneration and regeneration may potentially be utilized for the prevention and/or therapy of diabetic neuropathy (Balakumar et al.,

2009 for review). In this context, our finding showing that rimonabant treatment inhibits the overproduction of TNFα in spinal cord of diabetic mice supports the key role of such a blockade as a mechanism to counteract diabetic neuropathic pain.

4.5. Rimonabant restores NGF content in the sciatic nerve and improves axon morphology

Oxidative stress, especially in the peripheral nerves, has an important role in diabetes-induced impairment of neurotrophic support (Garrett et al., 1997) which is closely associated with Schwann cell injury (Kalichman et al., 1998) and nerve degeneration. We previously demonstrated a significant nerve regeneration after the repeated treatment with rimonabant in the chronic constriction injury model of neuropathy (Costa et al., 2005). In fact, the histological evaluation of sciatic nerve from rats treated with the compound revealed a morphology similar to that of control animals, with many myelinated fibers of small diameter, sign of regenerative process. In the light of the pivotal role of NGF in regenerative process of peripheral nerves and the strong support for the hypothesis that reduced levels or activity of NGF play a significant role in the pathogenesis of diabetic neuropathy (Pittenger and Vinik, 2003), we postulate an involvement of this neurotrophic factor in rimonabant-induced antiallodynia. Accordingly, we found low level of NGF in the sciatic nerve of diabetic mice and this decrease could be due to either reduced production or transport of NGF or both, possibly as a result of glucose-induced oxidative stress (Pittenger and Vinik, 2003). Unfortunately, clinical trials of NGF therapy in diabetic patients have not been successful (Apfel et al., 2000), in part because of the limitation in the exogenous NGF delivery and tolerability. We have reported here that the NGF content in the sciatic nerve of diabetic rats was restored to normal following the repeated treatment with rimonabant. Consequently to the rimonabant-induced improvement of the peripheral environment, histology of sciatic nerves revealed an increase in the normal fibers as compared with vehicle-treated mice, sign of some degree of nerve regeneration induced by the repeated treatment with rimonabant.

5. Conclusions

In conclusion, rimonabant is effective in relieving neuropathic pain associated with diabetes through a mechanism unrelated to blood glucose control. Besides a modulator effect upon TRPV1 receptor induced through a blockade of the plausible cannabinoid complimentary pathway, the relief of pain elicited by rimonabant might be ascribed to the ability of the compound to counteract oxidative stress in peripheral nerve, to inhibit TNFα overproduction in the spinal cord and to increase the NGF support, even if the exact molecular mechanism underlying such effects is still unknown and deserves further investigations. The data presented herein support the hypothesis that CB_1 antagonists would represent a new opportunity for diabetic patients in which the CB_1 receptor blockade has been already shown to increase insulin sensitivity and to reduce HbA_{1c}. Concerning rimonabant in particular, unfortunately, the increased incidence of depression and suicidality in patients tacking it as an anti-obesity agent, recently prompted the European Medicines Agency to halt sales of this drug. However, rimonabant remains an extremely valuable pharmacological tool to explore the therapeutic potential of CB_1 blockers. The hope is that neutral CB_1 receptor antagonists as well as allosteric modulators might share the anti-obesity and anti-diabetic effects of rimonabant, included that on diabetic neuropathy showed here by us, but with different side-effect profiles.

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