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Spinal nerve ligation reduces nitric oxide synthase activity and expression: Effect of resveratrol

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ABSTRACT

The effect of resveratrol on activity and expression of nitric oxide synthase (NOS) in the spinal cord of neuropathic rats was assessed. Spinal nerve ligation produced tactile allodynia along with a reduction of catalytic activity of the constitutive Ca^{2+}-dependent NOS (eNOS and nNOS isoforms) in the ipsilateral dorsal horn, but not contralateral dorsal or ipsilateral or contralateral ventral, spinal cord at 1, 5, 10 and 15 days after surgery compared to naïve and sham-operated animals. Nerve ligation also induced a reduction of nNOS expression in the ipsilateral dorsal horn spinal cord at 10 and 15 days after surgery. Intrathecal resveratrol reduced allodynia and reversed the reduction of constitutive Ca^{2+}-dependent NOS activity in the ipsilateral dorsal spinal cord. Moreover, resveratrol significantly reversed the reduction of nNOS expression in the ipsilateral dorsal horn spinal cord. Results show that spinal nerve ligation leads to development of tactile allodynia along with a reduction in constitutive Ca^{2+}-dependent NOS activity and nNOS isoform expression in the ipsilateral dorsal horn. Data suggest that resveratrol may reduce tactile allodynia in neuropathic rats by restoring altered NOS activity and expression.

1. Introduction

Peripheral nerve injury may lead to neuropathic syndromes characterized by spontaneous and evoked painful sensations. An exaggerated response to otherwise innocuous tactile stimuli (allodynia) is considered a hallmark and the most troublesome of these syndromes (Koltzenburg et al., 1994). The molecular mechanisms of neuropathic pain states are not clear. However, one consequence of nerve injuries is the appearance of adaptive changes in the expression of a variety of receptors, channels, and enzymes in the dorsal root ganglion of the injured nerve and in spinal neurons receiving input from injured afferents.

Changes in the spinal nitric oxide (NO) production may contribute to allodynia after nerve injury. Spinal NO release is evoked by NMDA receptor activation (Snyder, 1992; Montague et al., 1994; Sakai et al., 1998) and NO has been shown to enhance the release of excitatory amino acids (Akira et al., 1994; Montague et al., 1994; Bogdanov and Wurtman, 1997). Accordingly, spinal administration of NMDA receptor antagonists reduces tactile allodynia (Chaplan et al., 1997; Siegan et al., 1997). In addition, pharmacological evidence has suggested a correlation between altered NO spinal production and the generation and/or maintenance of neuropathic pain (Meller et al., 1992; Yamamoto and Shimoyama, 1995; Inoue et al., 1998; Naik et al., 2006). However, so far the role of spinal NO in the regulation of nociceptive processing after peripheral nerve injury is controversial, showing pronociception (see above), antinociception (Pan et al., 1998; Chen et al., 2000) or no change (Luo et al., 1999; Lee et al., 2005).

On the other hand, our group (Mixcoatl-Zecuatl et al., 2006; Bermúdez-Ocaña et al., 2006) and others (Chen et al., 2001; Chen and Pan, 2003) have reported that drugs which activate the NO synthesis reduce tactile allodynia in rats. Resveratrol (3,4′,5-trihydroxystilbene), a naturally occurring phytoalexin present in grapes and wine, has shown to increase activity and expression (Wallerath et al., 2002, 2005) of endothelial nitric oxide synthase (eNOS) isoform. In addition, data from our laboratory have recently shown that resveratrol is able to reduce tactile allodynia in an L-NAME-sensitive manner in neuropathic rats (Bermúdez-Ocaña et al., 2006). Taken together, these data suggest that resveratrol-induced antiallodynic effect may be related to the activity or expression of NOS in the spinal cord.

In this study we have examined the role of NO in L5/L6 spinal nerve injury-induced tactile allodynia by assessing the activity and expression of spinal NOS. In addition, we tested whether or not intrathecal resveratrol is able to modify tactile allodynia and spinal nerve injury-induced changes in activity and expression of NOS in the spinal cord.

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2. Materials and methods

2.1. Animals

Female Wistar rats aged 6–7 weeks (weight range, 140–160 g) from our own breeding facilities were used in this study. Previous studies from our laboratory have found no differences in tactile allodynia between female and male rats (Caram-Salas et al., 2007). Animals had free access to food and drinking water before experiments. Efforts were made to minimize animal suffering and to reduce the number of animals used. Rats were used once only. Experiments were carried out at the same hours of the day (10:00–16:00 h). All experiments followed the Guidelines on Ethical Standards for Investigation of Experimental Pain in Animals (Zimmermann, 1983). Additionally, the Institutional Animal Care and Use Committee approved the study.

2.2. Measurement of tactile allodynia

Rats were prepared according to the method of Kim and Chung (1992). Animals were anesthetized with a mixture of ketamine/xylazine (45:12 mg/kg, i.p.). After surgical preparation and exposure of the dorsal vertebral column, the left L5 and L6 spinal nerves were exposed and tightly ligated with 6-0 silk suture distal to the dorsal root ganglion. For sham-operated rats, the nerves were exposed but not ligated. After closing the incisions, animals were allowed to recover for 15 days. Rats exhibiting motor deficiency (such as paw-dragging) were discarded from the study (less than 5%).

Tactile allodynia was determined according to a previously reported method (Chaplan et al., 1994). Briefly, rats were transferred to a clear plastic, wire mesh-bottomed cage and allowed to acclimatize for 30 min. Von Frey filaments (Stoelting, Wood Dale, IL) were used to determine the 50% paw withdrawal threshold using the up–down method of Dixon (1980). A series of filaments, starting with one that had a buckling weight of 2 g, was applied in consecutive sequence to the plantar surface of the right hind paw with a pressure causing the filament to buckle. Lifting of the paw indicated a positive response and prompted the use of the next weaker filament whereas that absence of a paw withdrawal after 5 sec indicated a negative response and prompted the use of the next filament of increasing weight. This paradigm continued until four more measurements had been made after the initial change of the behavioral response or until 5 consecutive negative (assigned a score of 15 g) or four consecutive positive (assigned a score of 0.25 g) responses had occurred. The resulting scores were used to calculate the 50% response threshold by using the formula: 50% g threshold = $10^{(Xf+\kappa\partial)}$ / 10,000, where Xf = the value (in log units) of the final von Frey filament used, κ = the value (from table in Chaplan et al., 1994) for the pattern of positive and/or negative responses, and ∂ = the mean difference (in log units) between stimulus strengths. Behavioral tests were performed immediately before and 30 min after drug administration. Threshold was then assessed every 30 min until 5 h. Allodynia was considered to be present when paw withdrawal thresholds were <4 g. Rats not demonstrating allodynia were not further studied (less than 5%).

2.3. Spinal surgery

Ten days after the first surgery rats were anesthetized with a ketamine/xylazine mixture (45/12 mg/kg, i.p.) and placed in a stereotaxic head holder in order to expose the atlantooccipital membrane (Yaksh and Rudy, 1976). After piercing the membrane, a PE-10 catheter (7.5 cm) was passed intrathecally to the level of the thoracolumbar junction and the wound sutured. Rats were allowed to recover from surgery for at least 4–5 days in individualized cages before use. Animals showing any signs of motor impairment were euthanized with CO_2.

2.4. Drugs

Resveratrol was obtained from Sigma (St. Louis, MO, USA) and dissolved in 100% dimethyl sulfoxide.

2.5. Experimental protocols

Three groups of animals were employed. The first group of rats was used to determine the time course (0–15 days) of tactile allodynia in sham and L5/L6 spinal nerve-ligated rats. In the second group, the left L5/L6 spinal nerves were exposed and tightly ligated. Animals were assessed for tactile allodynia and then sacrificed and spinal cord harvested at day 1, 5, 10 and 15 following nerve injury. This group was compared with naïve and sham-operated rats. The third group of rats received a spinal injection of resveratrol (300 µg in 10 µl) at day 15 and animals were sacrificed and spinal cord harvested at 1, 2, 2.5, 3, 4 and 5 h later. Selection of the resveratrol dose was based on previous studies with this drug in the same model (Bermúdez-Ocaña et al., 2006).

2.6. Sample dissection

At day 1, 5, 10 and 15 following nerve injury, the nerve-ligated rats destined to the study of NOS catalytic activity and expression were re-anesthetized. The spinal cord segments L1–S1 were excised and the spinal cord was quickly extruded into an ice-cold isotonic saline solution and cleaned from surrounding tissue. The ventral horns were gently marked unilaterally by a scalpel incision to enable the ipsilateral (lesioned) and contralateral (unlesioned) sides to be identified. Excised tissues, which included L4–L6 spinal cord segments, were dropped into liquid nitrogen for 1 min and then stored in a freezer.

2.7. NOS radioassay

Catalytic activity of constitutive Ca^{2+}-dependent NOS (including, eNOS and nNOS isoforms), as well as the inducible Ca^{2+}-independent NOS (iNOS isoform) was determined by the conversion of [^{3}H]-L-arginine to [^{3}H]-L-citrulline according to the method of Bredt and Snyder (1990) with slight modifications previously described (Pérez-Severiano et al., 2002; Segovia and Pérez-Severiano, 2004). Each spinal cord tissue (ipsilateral and contralateral dorsal segment or ipsilateral and contralateral ventral segment) sample was homogenized in 250 µl of buffer (50 mM Tris–HCl, 0.1 mM EDTA, 0.1 mM EGTA, 0.1% β-mercaptoethanol, pH 7.5) containing a cocktail of protease inhibitors (100 µM leupeptin, 1 mM phenylmethylsulphonyl fluoride, 2 µg/ml aprotinin, 10 µg/ml soybean trypsin inhibitor) and 0.1% v/v Nonidet NP-40. A volume of homogenized solution containing 500 µg of protein was incubated for 30 min at 37 °C in the presence of 10 µM L-arginine-HCl, 0.2 µCi [^{3}H]-L-arginine, 1 mM NADPH, 100 nM calmodulin, 2.5 mM $CaCl_2$, and 30 µM BH_4. To assay the activity of Ca^{2+}-independent iNOS, the incubation was performed in the presence of 0.1 mM EGTA and 0.1 mM EDTA with no $CaCl_2$. Reactions were stopped by adding a buffer containing 2 mM EGTA, 2 mM EDTA, 20 mM HEPES, pH 5.5. The reaction mixture was applied onto a 1 ml column of cation interchange resin (Dowex-50W), which had been previously equilibrated with stop buffer. This column retains labeled arginine and allows [^{3}H]-L-citrulline to elute through the column. [^{3}H]-L-citrulline was quantified using a Beckman LS6500 scintillation counter. Results were expressed as ng [^{3}H]-L-citrulline/500 µg of protein per 30 min according to Pérez-Severiano et al. (2002).

2.8. nNOS expression

The assay has been previously described (Segovia et al., 1998). Briefly, we used the same homogenized samples that were employed in NOS activity measurement and the total protein was analyzed by the method of Lowry et al. (1951). Western blots were carried out

using 50 µg of protein per lane on an 8% SDS-polyacrylamide gel and transferred onto Hybond polyvinylidene fluoride membrane (PVDF, Amersham Biosciences, UK). After that, the PVDF membrane was blocked with TBS containing 5% skim milk and 0.05% Tween 20 for 1 h at room temperature. Then it was incubated overnight at 4 °C with polyclonal antibody against nNOS (Santa Cruz Biotechnology, CA, USA), at a final dilution of 1:250. The membrane was then washed and incubated with the secondary goat antimouse peroxidase-labeled antibody (Zymed) diluted 1:6000 in blocking solution for 1 h at room temperature. Membranes were extensively washed and bands were identified by chemoluminesence using the Luminol detection system (Santa Cruz Biotechnology, CA, USA). The relative presence of nNOS was normalized with the reference protein β-actin (Díaz-Barriga et al., 1989). Images from film were digitally acquired with a BioDoc-It System (UVP) and a densitometry analysis was performed using the Lab Works™ 4.0 Image Acquisition and Analysis Software (UVP). Data were expressed as normalized optical density (OD) in arbitrary units.

2.9. Data analysis and statistics

Data of the time course of tactile allodynia in sham and spinal nerve-ligated rats are the mean±S.E.M. for 6 animals per group. Results of activity and expression experiments are given as the mean± S.E.M. for 3–4 animals per group. Two-way analysis of variance (ANOVA) followed by the Bonferroni or Tukey's test were used to assess the effect of time (1, 5, 10 and 15 days) and spinal nerve ligation (versus naïve and sham groups) in the activity and expression experiments (SPSS software, version 13). One-way ANOVA was used to determine differences in the effect of resveratrol on activity and expression of NOS in dorsal horn of neuropathic rats. Differences were considered to reach statistical significance when $P<0.05$.

3. Results

3.1. Spinal nerve ligation

Ligation of L5/L6 spinal nerves produced tactile allodynia in the rats evidenced by a decrease in paw withdrawal threshold as compared to sham-operated rats. Tactile allodynia was evident 1 day after spinal nerve ligation and remained stable for 15 days (Fig. 1).

3.2. NOS activity and expression

Besides tactile allodynia, spinal nerve ligation produced a significant reduction ($P<0.05$) of catalytic activity of the constitutive Ca^{2+}-dependent NOS in the ipsilateral dorsal horn, but not contralateral dorsal or ipsilateral ventral (not shown), spinal cord at 1, 5, 10 and 15 days after surgery compared to naïve and sham-operated animals (Fig. 2). No changes in the activity of inducible Ca^{2+}-independent NOS were observed (data not shown). Moreover, nerve ligation also induced a significant reduction ($P<0.05$) of nNOS expression in the ipsilateral dorsal horn (Fig. 3), but not contralateral dorsal or ipsilateral or contralateral ventral (not shown), spinal cord at 10 and 15 days after surgery compared to naïve and sham-operated animals.

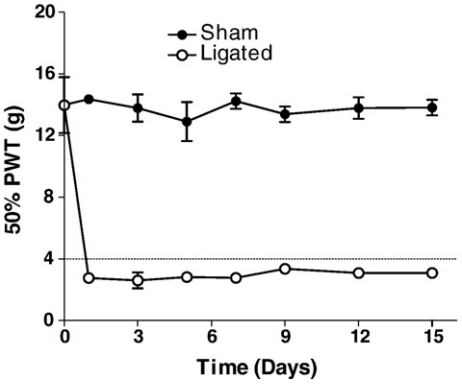

Fig. 1. Time course of tactile allodynia in rats with ligation of L5/L6 spinal nerves compared to sham-operated rats. Data are the mean±S.E.M. for six animals. PWT, paw withdrawal threshold.

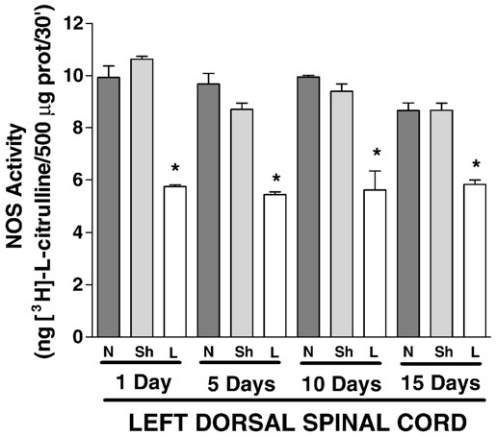

Fig. 2. Catalytic activity of the constitutive Ca^{2+}-dependent NOS in the left dorsal horn of naïve (N) and sham (Sh) rats as well as animals submitted to ligation (L) of L5/L6 spinal nerves. Rats were operated and spinal cords harvested at 1, 5, 10 and 15 days after surgery. Catalytic activity is expressed as ng of [^{3}H]-citrulline/500 µg protein/30 min. Bars are the mean±S.E.M. for 3–4 animals. * Significantly different from naïve and sham groups, as determined by two-way ANOVA followed by the Bonferroni test.

3.3. Effect of resveratrol on tactile allodynia and reduction of NOS activity and expression induced by spinal nerve ligation

Intrathecal treatment with resveratrol (300 µg, day 15), but not vehicle, increased withdrawal threshold and this was interpreted as a

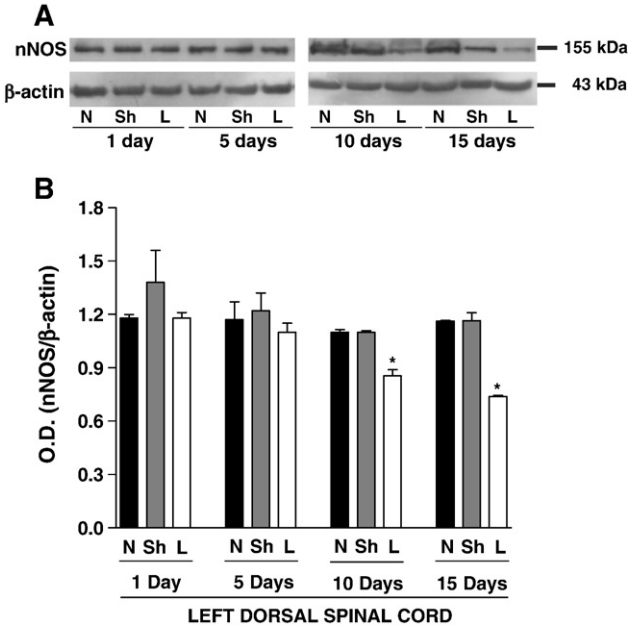

Fig. 3. Expression of nNOS isoform in naïve (N), sham (Sh) and ligated (L) rats. A) Immunoblots of nNOS (155 kDa) and β-actin (43 kDa) at different days after neuropathy induction. B) Expression of nNOS isoform. Data are expressed as the optical density (O.D.) of the nNOS/β-actin ratio in arbitrary units. Bars are the mean±S.E.M. for 3–4 animals. *Significantly different from naïve and sham groups, as determined by two-way ANOVA followed by the Bonferroni test.

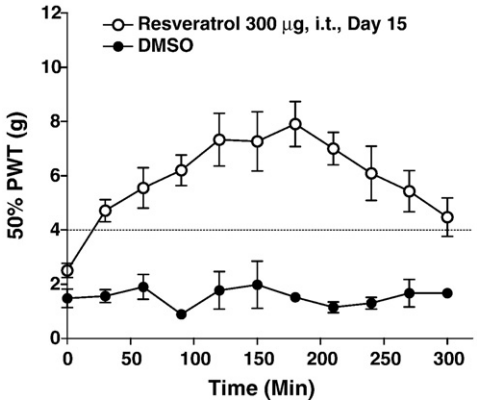

Fig. 4. Antiallodynic effect of resveratrol in rats submitted to ligation of L5 and L6 spinal nerves. Rats were treated with intrathecal vehicle or resveratrol (day 15) 30 min before starting thresholds evaluations. Data are expressed as the 50% paw withdrawal threshold (PWT). Data are the mean ± S.E.M. for six animals.

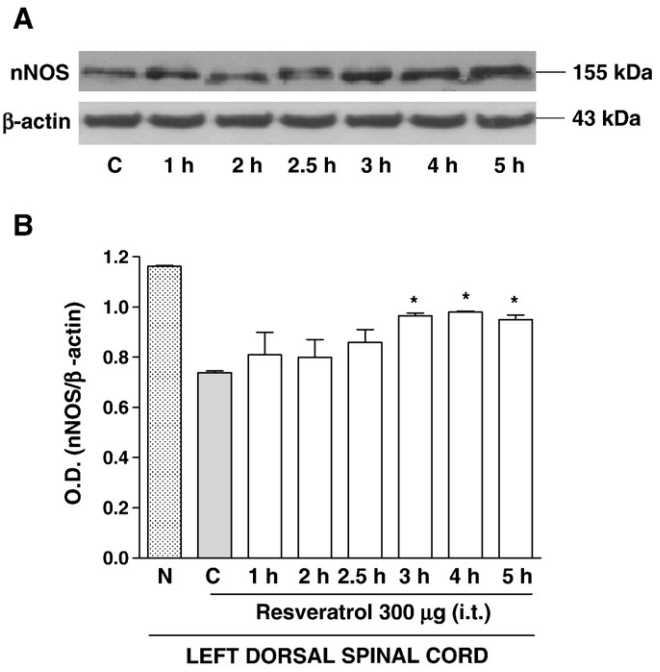

Fig. 6. Effect of resveratrol on the expression of the nNOS isoform in rats submitted to ligation of L5/L6 spinal nerves. A) Immunoblots of nNOS (155 kDa) and β-actin (43 kDa) at different times after resveratrol treatment. B) Effect of resveratrol on the expression of nNOS. Data are expressed as the optical density (O.D.) of the nNOS/β-actin ratio in arbitrary units. Bars are the mean ± S.E.M. for 3–4 animals. *Significantly different from control (C) group, as determined by one-way ANOVA followed by the Tukey's test. Naïve (N) group is plotted as reference.

reduction of tactile allodynia in neuropathic rats (Fig. 4). Besides its antiallodynic effect, resveratrol significantly reversed ($P<0.05$), above the baseline levels, the observed reduction of constitutive Ca^{2+}-dependent NOS activity in the left dorsal (ipsilateral, Fig. 5), but not right dorsal or left or right ventral horn (not shown), spinal cord compared to control rats. The reversal started at 2 h and it was maximal at about 3 h, time at which the maximal antiallodynic effect of resveratrol was reached. Resveratrol did not modify Ca^{2+}-independent NOS activity (data not shown). In addition, intrathecal injection of resveratrol partially but significantly reversed ($P<0.05$) the observed reduction of nNOS expression in the left dorsal horn (ipsilateral, Fig. 6), but not right dorsal (contralateral) or left or right ventral horn (not shown), spinal cord compared to control rats. Reversal was significant at 3 h and remained up to 5 h.

4. Discussion

Ligation of L5/L6 spinal nerves produced tactile allodynia from day 1 to 15. This allodynia was accompanied by a significant reduction of the constitutive Ca^{2+}-dependent NOS activity in the left dorsal horn (ipsilateral), but not right dorsal (contralateral) or left or right ventral, at day 1, 5, 10 and 15. Our results agree with previous observations

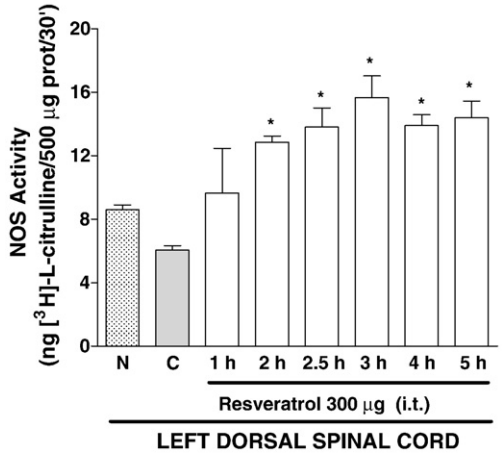

Fig. 5. Effect of resveratrol on the catalytic activity of the constitutive Ca^{2+}-dependent NOS in the left dorsal horn of rats submitted to ligation of L5 and L6 spinal nerves. Rats were operated, treated with resveratrol at day 15 and spinal cords were harvested after resveratrol treatment. Catalytic activity is expressed as ng of [^{3}H]-citrulline/500 µg protein/30 min. Bars are the mean ± S.E.M. for 3–4 animals. *Significantly different from control (C) group, as determined by one-way ANOVA followed by the Tukey's test. Naïve (N) group is plotted as reference.

showing a decrease in NOS activity in lumbar spinal cord 2 and 4 weeks after neuropathic surgery (Choi et al., 1996). The fact that the reduced activity of NOS was observed only in the left dorsal horn spinal cord, ipsilateral to nerve damage, supports a possible correlation with the spinal sensory processing of neuropathic pain. According to a reduced activity, nNOS expression was also reduced at day 10 and 15 after surgery. Again, this reduction was only observed in the left dorsal (ipsilateral), but not right dorsal or left or right ventral horn suggesting a possible relevance in neuropathic pain. Our results agree with those of Zhang et al. (1993) reporting a reduction in the number of NOS-immunoreactive neurons in lamina II of the left dorsal horn (ipsilateral). Other authors have reported no change in nNOS mRNA and protein expression in dorsal spinal cord 2 weeks after nerve injury (Luo et al., 1999). Contrariwise, an increase in activity (Choi et al., 1996; Cizkova et al., 2002), mRNA and protein expression (Luo et al., 1999) as well as the number of NOS-immunoreactive neurons (Fiallos-Estrada et al., 1993; Zhang et al., 1993; Steel et al., 1995; Luo et al., 1999; González-Hernández and Rustioni, 1999; Cizkova et al., 2002; Lukacova et al., 2003) was reported in dorsal root ganglion. Differences in the results are difficult to explain. One possibility is that nerve injury increases NOS activity and expression in dorsal root ganglion whereas in spinal cord, changes are in the opposite way. Whatever the case, it has been suggested that changes in dorsal root ganglion are not relevant for neuropathic pain, as nNOS up-regulation in this site remains elevated in nerve-injured rats fully recovered from tactile allodynia or in rats that do not develop tactile allodynia after nerve ligation (Luo et al., 1999). Another possibility is that changes induced by peripheral nerve injury (in this study) may affect NOS activity and expression only in intrinsic neurons in dorsal horn or descending fibers, but not dorsal root ganglion. Methods to induce neuropathic pain may also have a role in these differences. However, the final answer will need more investigation.

Previous observations of our group have shown that the spinal administration of resveratrol reduces tactile allodynia in a dose-

dependent and L-NAME-sensitive manner in rats submitted to L5/L6 spinal nerve ligation (Bermúdez-Ocaña et al., 2006). This study suggested that resveratrol produces its antiallodynic effect through activation of spinal NOS. Thus, in order to reduce tactile allodynia, resveratrol may affect NOS activity or expression or both. In the current study, these alternatives were examined. Intrathecal treatment with resveratrol (day 15) diminished tactile allodynia and reversed the observed reduction of constitutive Ca^{2+}-dependent NOS but not inducible Ca^{2+}-independent NOS activity in the left dorsal horn (ipsilateral) spinal cord compared to control rats. To the best of our knowledge, this is the first report concerning the effect of resveratrol on NOS activity in neuropathic rats. Results agree with data showing that either acute (2 min) or long-term exposition (24–72 h) to resveratrol is able to increase activity of eNOS in human umbilical vein endothelial cells (Wallerath et al., 2002, 2003; Leikert et al., 2002). In addition, resveratrol increases endothelial and iNOS activity in the human hepatocyte-derived cancer cell line HepG2 (Notas et al., 2006). Contrariwise, there is recent evidence that resveratrol is able to reduce thermal hyperalgesia in diabetic mice by reducing NO release as well as oxidative damage (Sharma et al., 2007; Ates et al., 2007; Kumar et al., 2007). Differences may be due to different model to induce nerve injury.

Besides its effect on allodynia and NOS activity, intrathecal administration of resveratrol (day 15) partially reversed spinal nerve ligation-induced reduction of nNOS protein expression in the left dorsal horn spinal cord. This is the first study assessing the effect of resveratrol on the spinal nNOS in rats submitted to spinal nerve injury. However, data from cell cultures show that resveratrol induces eNOS protein expression in human umbilical vein endothelial cells (Leikert et al., 2002). There are also studies reporting that resveratrol induces the expression of iNOS mRNA in mice (Imamura et al., 2002). Then, our results suggest that, besides induction of eNOS expression, resveratrol could be able to induce expression of the neuronal isoform at least in the spinal cord. The mechanisms by which resveratrol increases expression of nNOS on the spinal cord of neuropathic rats are unknown and they warrant further investigation.

In summary, this study has shown that spinal nerve ligation leads to development of tactile allodynia in rats. Allodynia is accompanied by a reduction in constitutive Ca^{2+}-dependent NOS activity and a reduction of nNOS isoform expression in the left dorsal horn, ipsilateral to the nerve damage. Intrathecal administration of resveratrol reduces tactile allodynia and it reverses spinal nerve ligation-induced reduction in NOS activity and nNOS expression. Thus data suggest that resveratrol may reduce tactile allodynia in neuropathic rats by restoring altered NOS activity and expression.

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References

Akira T, Henry D, Wasterlain CG. Nitric oxide mediates the sustained opening of NMDA receptor-gated ionic channels which follows transient excitotoxic exposure in hippocampal slices. Brain Res 1994;652:190–4.

Ates O, Cayli SR, Yucel N, Altinoz E, Kocak A, Durak MA, et al. Central nervous system protection by resveratrol in streptozotocin-induced diabetic rats. J Clin Neurosci 2007;14:256–260.

Bermúdez-Ocaña DY, Ambriz-Tututi M, Pérez-Severiano F, Granados-Soto V. Pharmacological evidence for the participation of NO-cyclic GMP-PKG-K^+ channel pathway in the antiallodynic action of resveratrol. Pharmacol Biochem Behav 2006;84:535–42.

Bogdanov MB, Wurtman RJ. Possible involvement of nitric oxide in NMDA-induced glutamate release in the rat striatum: an in vivo microdialysis study. Neurosci Lett 1997;221:197–201.

Bredt DS, Snyder SH. Isolation of nitric oxide synthetase, a calmodulin-requiring enzyme. PNAS 1990;87:682–5 1990.

Caram-Salas NL, Reyes-García G, Bartoszyk GD, Araiza-Saldaña CI, Ambriz-Tututi M, Rocha-González HI, et al. Subcutaneous, intrathecal and periaqueductal grey administration of asimadoline and ICI-204448 reduces tactile allodynia in the rat. Eur J Pharmacol 2007;573:75–83.

Chaplan SR, Bach RW, Pogrel JW, Chung JM, Yaksh TL. Quantitative assessment of tactile allodynia in the rat paw. J Neurosci Methods 1994;53:55–63.

Chaplan SR, Malmberg AB, Yaksh TL. Efficacy of spinal NMDA receptor antagonism in formalin hyperalgesia and nerve injury evoked allodynia in the rat. J Pharmacol Exp Ther 1997;280:829–38.

Chen SR, Eisenach JC, Pan HL. Intrathecal *S*-nitroso-*N*-acetylpenicillamine and L-cysteine attenuate nerve injury-induced allodynia through noradrenergic activation in rats. Neuroscience 2000;101:759–65.

Chen SR, Khan GM, Pan HL. Antiallodynic effect of intrathecal neostigmine is mediated by spinal nitric oxide in a rat model of diabetic neuropathic pain. Anesthesiology 2001;95:1007–12.

Chen SR, Pan HL. Spinal nitric oxide contributes to the analgesic effect of intrathecal [D-pen2, d-pen5]-enkephalin in normal and diabetic rats. Anesthesiology 2003;98:217–22.

Choi Y, Raja SN, Moore LC, Tobin JR. Neuropathic pain in rats is associated with altered nitric oxide synthase activity in neural tissue. J Neurol Sci 1996;138:14–20.

Cizkova D, Lukacova N, Marsala M, Marsala J. Neuropathic pain is associated with alterations of nitric oxide synthase immunoreactivity and catalytic activity in dorsal root ganglia and spinal dorsal horn. Brain Res Bull 2002;58:161–71.

Diaz-Barriga F, Hernández JM, Carrizales L, Domínguez MC, Yañez L, Palmer E, et al. Interactions of cadmiun with actin microfilaments. Toxicol In vitro 1989;3:277–84.

Dixon WJ. Efficient analysis of experimental observations. Annu Rev Pharmacol Toxicol 1980;20:441–62.

Fiallos-Estrada CE, Kummer W, Mayer B, Bravo R, Zimmermann M, Herdegen T. Long-lasting increase of nitric oxide synthase immunoreactivity, NADPH-diaphorase reaction and c-JUN co-expression in rat dorsal root ganglion neurons following sciatic nerve transection. Neurosci Lett 1993;150:169–73.

González-Hernández T, Rustioni A. Nitric oxide synthase and growth-associated protein are co-expressed in primary sensory neurons after peripheral injury. J Comp Neurol 1999;404:64–74.

Imamura G, Bertelli AA, Bertelli A, Otani H, Maulik N, Das DK. Pharmacological preconditioning with resveratrol: an insight with iNOS knockout mice. Am J Physiol Heart Circ Physiol 2002;282:H1996–2003.

Inoue T, Mashimo T, Shibata M, Shibuta S, Yoshiya I. Rapid development of nitric oxide-induced hyperalgesia depends on an alternate to the cGMP-mediated pathway in the rat neuropathic pain model. Brain Res 1998;792:263–70.

Kim SH, Chung JM. An experimental model for peripheral neuropathy produced by segmental spinal nerve ligation in the rat. Pain 1992;50:355–63.

Koltzenburg M, Torebjork HE, Wahren LK. Nociceptor modulated central sensitization causes mechanical hyperalgesia in acute chemogenic and chronic neuropathic pain. Brain 1994;117:579–91.

Kumar A, Kaundal RK, Iyer S, Sharma SS. Effects of resveratrol on nerve functions, oxidative stress and DNA fragmentation in experimental diabetic neuropathy. Life Sci 2007;80:1236–44.

Lee DH, Singh JP, Lodge D. Experiments with nitric oxide synthase inhibitors in spinal nerve ligated rats provide no evidence of a role for nitric oxide in neuropathic mechanical allodynia. Neurosci Lett 2005;385:179–83.

Leikert JF, Rathel TR, Wohlfart P, Cheynier V, Vollmar AM, Dirsch VM. Red wine polyphenols enhance endothelial nitric oxide synthase expression and subsequent nitric oxide release from endothelial cells. Circulation 2002;106:1614–7.

Lowry OH, Rosenbrough NJ, Farr L, Randall LJ. Protein measurement with the folin phenol reagent. J Biol Chem 1951;193:265–75.

Lukacova N, Cizkova D, Krizanova O, Pavel J, Marsala M, Marsala J. Peripheral axotomy affects nicotinamide adenine dinucleotide phosphate diaphorase and nitric oxide synthases in the spinal cord of the rabbit. J Neurosci Res 2003;71:300–13.

Luo ZD, Chaplan SR, Scott BP, Cizkova D, Calcutt NA, Yaksh TL. Neuronal nitric oxide synthase mRNA upregulation in rat sensory neurons after spinal nerve ligation: lack of a role in allodynia development. J Neurosci 1999;19:9201–8.

Meller ST, Pechman PS, Gebhart GF, Maves TJ. Nitric oxide mediates the thermal hyperalgesia produced in a model of neuropathic pain in the rat. Neuroscience 1992;50:7–10.

Mixcoatl-Zecuatl T, Flores-Murrieta FJ, Granados-Soto V. The nitric oxide-cyclic GMP-protein kinase G-K^+ channel pathway participates in the antiallodynic effect of spinal gabapentin. Eur J Pharmacol 2006;531:87–95.

Montague PR, Gancayco CD, Winn MJ, Marchase RB, Friedlander MJ. Role of NO production in NMDA receptor-mediated neurotransmitter release in cerebral cortex. Science 1994;263:973–7.

Naik AK, Tandan SK, Kumar D, Dudhgaonkar SP. Nitric oxide and its modulators in chronic constriction injury-induced neuropathic pain in rats. Eur J Pharmacol 2006;530:59–69.

Notas G, Nifli AP, Kampa M, Vercauteren J, Kouroumalis E, Castanas E. Resveratrol exerts its antiproliferative effect on HepG2 hepatocellular carcinoma cells, by inducing cell cycle arrest, and NOS activation. Biochim Biophys Acta 2006;1760:1657–66.

Pan HL, Chen SR, Eisenach JC. Role of spinal NO in antiallodynic effect of intrathecal clonidine in neuropathic rats. Anesthesiology 1998;89:1518–23.

Pérez-Severiano F, Escalante B, Vergara P, Ríos C, Segovia J. Age-dependent changes in nitric oxide synthase activity and protein expression in striata of mice transgenic for the Huntington's disease mutation. Brain Res 2002;951:34–42.

Sakai M, Minami T, Hara N, Nishihara I, Kitade H, Kamiyama Y, et al. Stimulation of nitric oxide release from rat spinal cord by prostaglandin E_2. Br J Pharmacol 1998;123:890–4.

Segovia J, Pérez-Severiano F. Oxidative damage in Huntington's disease. Methods Mol Biol 2004;277:321–34.

Segovia J, Vergara P, Brenner M. Differentiation-dependent expression of transgenes in engineered astrocyte cell lines. Neurosci Lett 1998;242:172–6.

Sharma S, Kulkarni SK, Chopra K. Effect of resveratrol, a polyphenolic phytoalexin, on thermal hyperalgesia in a mouse model of diabetic neuropathic pain. Fundam Clin Pharmacol 2007;21:89–94.
Siegan JB, Hama AT, Sagen J. Suppression of neuropathic pain by a naturally-derived peptide with NMDA antagonist activity. Brain Res 1997;755:331–4.
Snyder SH. Nitric oxide: first in a new class of neurotransmitters. Science 1992;257:494–6.
Steel JH, Terenghi G, Chung JM, Na HS, Carlton SM, Polak JM. Increased nitric oxide synthase immunoreactivity in rat dorsal root ganglia in a neuropathic pain model. Neurosci Lett 1994;169:81–4.
Wallerath T, Deckert G, Ternes T, Anderson H, Li H, Witte K, et al. Resveratrol, a polyphenolic phytoalexin present in red wine, enhances expression and activity of endothelial nitric oxide synthase. Circulation 2002;106:1652–8.
Wallerath T, Li H, Gödtel-Ambrust U, Schwarz PM, Förstermann U. A blend of polyphenolic compounds explains the stimulatory effect of red wine on human endothelial NO synthase. Nitric Oxide 2005;12:97–104.
Wallerath T, Poleo D, Li H, Forstermann U. Red wine increases the expression of human endothelial nitric oxide synthase: a mechanism that may contribute to its beneficial cardiovascular effects. J Am Coll Cardiol 2003;41(3):471–8.
Yaksh TL, Rudy TA. Chronic catheterization of the spinal subarachnoid space. Physiol Behav 1976;17:1031–6.
Yamamoto T, Shimoyama N. Role of nitric oxide in the development of thermal hyperesthesia induced by sciatic nerve constriction injury in the rat. Anesthesiology 1995;82:1266–73.
Zhang X, Verge V, Wiesenfeld-Hallin Z, Ju G, Bredt D, Synder SH, et al. Nitric oxide synthase-like immunoreactivity in lumbar dorsal root ganglia and spinal cord of rat and monkey and effect of peripheral axotomy. J Comp Neurol 1993;335:563–75.
Zimmermann M. Ethical guidelines for investigations on experimental pain in conscious animals. Pain 1983;16:109–10.