TRPA1 receptor modulation attenuates bladder overactivity induced by spinal cord injury

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Spinal cord injury (SCI) impairs micturition by interrupting the communication between the cerebral and spinal circuits that coordinate bladder and urethral activities (38, 39). After a period of bladder areflexia, spontaneous and involuntary bladder contractions, which characterize the overactive bladder (OAB), are triggered by the emergence of a micturition reflex at the spinal level. Concomitantly to OAB, bladder-sphincter dyssynergia is induced, resulting in inefficient voiding (11). The reorganization of bladder reflex pathways seems to be mediated, in part, by bladder afferent C-fibers (12). Therefore, the suppression of bladder afferent nerves hyperexcitability is hypothesized to be effective in OAB symptoms improvement.

Studies have been carried out to identify the mechanisms that control bladder afferent activity. Several receptors have been identified in neuronal and nonneuronal structures of the bladder as potential candidate molecules in the modulation of its sensorial function, among them is the transient receptor potential vanilloid 1 (TRPV1). TRPV1 is a nonspecific ion channel activated by heat, protons (33), and vanilloids such as capsaicin and endovanilloids such as anandamide (43). The expression of TRPV1 is firmly established in sensory fibers of the bladder (3–5, 7, 8, 42). Moreover, some studies suggest that TRPV1 is also localized in structures nonneuronal of the bladder, such as urothelium (3, 4, 7, 10, 20), smooth muscle, and interstitial cells (24). Nevertheless, the localization of TRPV1 in nonneuronal cells of the bladder is still unclear due to the controversial findings that do not show the presence of TRPV1 in bladder urothelium (37, 42). Reinforcing this disagreement, a nonspecific cellular TRPV1 immunoreactivity in urothelium from TRPV1 knockout mice was observed (15). Therefore, the literature data might be interpreted carefully, taking account the antibody, species, and technique used in the study.

The possible presence of TRPV1 in urothelium is of particular interest since that this tissue exhibits specialized sensory and signaling properties (7). The increase of TRPV1 expression is associated with upregulation of the urothelial functions, such as enhanced release of chemical mediators and neurotransmitters, which have been suggested to influence afferent nerve activity and to contribute to OAB symptoms (40). Intravesical administration of the TRPV1 activators, namely capsaicin or resiniferatoxin, which desensitize afferent neurons, has been introduced into clinical practice for OAB treatment. Nevertheless, this therapy is frequently associated with suprapubic burning and/or painful sensations. Moreover, a significant fraction of patients do not respond to capsaicin or resiniferatoxin therapy, stressing the need to search for further targets in the modulation of bladder sensory nerves (21).

Currently, another ion channel has been focused in bladder activity. The ankyrin-repeat transient receptor potential 1 (TRPA1) was initially characterized as a thermoreceptor activated by noxious cold, coexpressed with the TRPV1 receptor in subsets of sensory neurons (30). It is localized in sensory nerves that innervate the rodents’ bladder (23, 31) and in the epithelium of both rat and human bladder (13, 31) and is upregulated in the bladder mucosa of patients with bladder outlet obstruction (13). We previously reported that TRPA1 mediates the contractile response caused by allyl isothiocyanate and cinnamaldehyde in rat bladder (2). Moreover, it was

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been demonstrated that the intravesical administration of these TRPA1 agonists causes bladder hyperreflexia through a C fiber-mediated afferent pathway (14, 31).

Bearing in mind the studies showing a link between TRPA1 expression/activation and bladder disorders, the present study was designed to assess the role exerted by TRPA1 on OAB following SCI.

EXPERIMENTAL PROCEDURES

Subjects. Adult male Wistar rats (10 wk old and weighing 270–300 g), obtained from the animal house of the Department of Pharmacology, Federal University of Santa Catarina (UFSC, Florianópolis, Brazil), were used throughout the experiments. The animals were housed in a room maintained at a constant temperature of 22 ± 2°C and under a 12:12-h light-dark cycle at 60–80% humidity with food and water available ad libitum. All procedures were approved by the UFSC Ethics Committee (process number PP 000156) and they are in accordance with the National Institutes of Health (NIH) Animal Care Guidelines (NIH Publications #80–23).

Surgical procedure. The SCI procedure used was previously described (34) with some minor modifications. Animals were previously treated with a wide spectrum antibiotic (300 mg/kg im oxytetracyclin chloride). The surgical procedure was performed under anesthesia with a 1:1 mixture of xylazine (10 mg/kg) and ketamine (70 mg/kg) given by intraperitoneal route. A 2-cm dorsal midline incision was made and the vertebral body was exposed. The paraspinal muscles were dissected from the T9-T12 spinous processes and moved laterally with the aid of a forceps. After removal of spinous process at the T10-T11 vertebra level, a small hole (1.5-mm diameter) was done in the T11 vertebra level using a dental drill. The hole allowed the visualization of the spinal cord with the intact dura and the insertion of an embolectomy catheter (2-French Fogarty; Lemaître Catheters, Burlington, VT).

Before each surgical procedure, the catheter diameter was calibrated (4.5 mm) with aid of a caliper and a syringe previously filled with water. Following the calibration, the catheter was inserted and advanced cranially for 1 cm, so that the center of the balloon rested at the T10 vertebra level. Then, the balloon was gently inflated until reaching a diameter of 4.5 mm, maintained until the disappearance of spinal reflexes, deflated, and slowly removed. Muscle and skin were sutured in anatomical layers. Sham-operated animals were submitted to the same protocol; however, the catheter was not inserted into the epidural space.

During the first 15 to 20 days post-SCI, urinary bladders were manually emptied twice daily, until partial micturition restoration. The animals’ care before and after the surgical procedure followed the protocol (25) of the Multicenter Animal Spinal Cord Injury Study (MASCIS).

The animals were divided into three experimental groups: naive (no surgical intervention), sham (surgical intervention, without catheter insertion), and SCI (surgical intervention with catheter insertion). Catheter placement for drug intrathecal delivery. To obtain a sustained drug intrathecal delivery, a soft polyurethane catheter (Alzet Durect, Cupertino, CA) was inserted through a small hole in the dura at T11 vertebra level below the injury site, soon after the surgical procedure. The tip of the catheter was positioned subdurally on the dorsal side of the spinal cord over the center of injury and reached L6-S1 spinal cord level. The catheter was tied to the muscles with some stitches and the other tip of the catheter was sealed and left subcutaneously. Muscles and skin were sutured. The same procedure was performed for the sham group.

To evaluate the bladder contractility (in vitro), on day 7 after surgery the animals were anesthetized with isoflurane (2%) and a small skin incision was performed to access the catheter. Next, an osmotic pump (Alzet type 2001), operating at a rate of 1 μl/h for 7 days, was filled with TRPA1 antisense (AS-ODN: 5′-TCTATGGGGTGGTGGTGG-3′; 0.5 nmol/μl) or TRPA1 mismatch (MM-ODN: 5′-ACTACTACACTAGACTAC-3′; 0.5 nmol/μl; Biognostik, Göttingen, Germany) and attached to the catheter. To evaluate the voiding behavior (in vivo), on day 14 after surgery the animals were submitted to the same procedure above and an osmotic pump operating at a rate of 0.5 μl/h for 14 days (Alzet type 2002) was attached to the catheter. The osmotic pump was placed between the shoulder blades. The animals were euthanized on days 14 or 28 after surgery.

Behavioral assessment. The Basso, Beattie, and Bresnahan (BBB) behavioral test was performed to measure the functional recovery of the rats’ hindlimbs as described (6). The scale used for measuring hindlimb function after SCI ranges from 0, indicating no spontaneous movement, to a maximum score of 21, indicating normal motor function. An increasing score indicates the use of individual joints, coordinated joint movement, coordinated limb movement, weight-bearing and other functions. To perform this behavior test, rats were first adapted to the open field. The BBB test was carried out every 2 days from day 0 to day 28 following surgical procedure.

Histopathological analysis. Animals were euthanized by pentobarbital sodium overdose (100 mg/kg ip). Bladders were carefully removed 2, 7, 14, and 28 days after SCI and weighed as an indicator of bladder hypertrophy. To assess the bladder histopathological alterations, tissue samples were collected in the same periods and fixed in a PBS solution containing 4% paraformaldehyde for 24 h at room temperature. Following fixation, tissue samples were embedded in paraffin, sectioned (5-μm slices), and stained with hematoxylin and eosin.

In another set of experiments, the neutrophil infiltration into the urinary bladder was assessed indirectly by measuring the myeloperoxidase (MPO) activity. For this purpose, bladders were homogenized in 5% ethylenediamine tetraacetic acid/NaCl buffer (pH 4.7) and centrifuged at 10,000 g for 15 min at 4°C. The pellet was resuspended in 0.5% hexadecyl trimethyl ammonium bromide buffer (pH 5.4) and samples were frozen in liquid nitrogen and thawed three times. Upon being thawed, the samples were similarly centrifuged, and 25 μl of the supernatant were used for the MPO assay. The MPO enzymatic reaction was assessed by addition of 1.6 mM tetramethylbenzidine, 80 mM NaPO₄, and 0.3 mM hydrogen peroxide (H₂O₂). Total absorbance was measured with a spectrophotometer at 690 nm and the results were expressed in optical density per milligram of tissue.

Quantitative real-time PCR. Bladder, dorsal root ganglion (DRG; L6-S1), and the corresponding segment of the spinal cord were removed 2, 7, and 14 days after surgery. Total RNA from the tissues was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol. RNA concentration was determined by NanoDrop 1100 (Nanodrop Technologies, Wilmington, DE). Reverse transcription assay was carried out with the M-MLV Reverse Transcriptase (Invitrogen) according to the manufacturer’s instructions. cDNA was amplified in duplicate using TaqMan Universal PCR Master Mix Kit (Applied Biosystems, Foster City, CA) with specific TaqMan gene expression target genes (Applied Biosystems), the 3′ quencher MGB, and FAM-labeled probe for rat TRPA1 (Rn01473803_m1) and 3′ quencher MGB and VIC-labeled probe for rat β-actin ACTB (Rn00667869_m1), which was used as an endogenous control for normalization. The PCR reactions were performed in a 96-well optical reaction plate (Applied Biosystems). The thermal cycler parameters were as follows: 50°C for 2 min, 95°C for 10 min, 55 cycles of 95°C for 15 s, and 60°C for 1 min. Expression of the target genes was calibrated against conditions found in naive animals.

Immunohistochemical analysis. On days 2, 7, 14, and 28 after surgery, immunohistochemical detection of TRPA1 was carried out in the bladder, DRG (L6-S1), and in corresponding segment of the spinal cord slices (5 μm) using rabbit anti-TRPA1 polyclonal antibody (1:100; Abcam, Cambridge, MA). High-temperature antigen retrieval was performed by immersion of the slices in a water bath at 95–98°C.
in 10 mmol/l trisodium citrate buffer, pH 6.0, for 40 min. After overnight incubation with primary antibody at 4°C, the slides were washed with PBS and incubated with appropriate biotin-coupled secondary antibody (1:250; DakoCytomation, Carpenteria, CA) for 1 h at room temperature. The negative control consisted of the replacement of primary antibody by nonimmune rabbit serum (Abcam) in equivalent concentration. The sections were then washed in PBS and incubated with streptavidin-peroxidase (1:250; Invitrogen) for 1 h. The visualization was completed by the use of 3,3'-diaminobenzidine (DakoCytomation) in chromogen solution and light counterstaining with Harris’s hematoxylin solution (Mercck, Darmstadt, Germany). Images were obtained with a microscope (Eclipse 50i; Nikon, Melville, NY) and Digital Sight Camera (DS-Fi1; Nikon). Settings for image acquisition were identical for the control and experimental tissues. For each tissue, five images were obtained. The images were transferred to a computer, and the average pixel color intensity of TRPA1 staining was calculated for each image using NIH ImageJ 1.36b imaging software (NIH, Bethesda, MD) and the results were represented as arbitrary units per area. The remaining value was divided by the surrounded area. The results were represented as arbitrary units per area.

Preparation of membrane extract. Bladders were collected on days 2, 7, 14, and 28 after surgery, snap-frozen in liquid nitrogen, and homogenized in lyses buffer containing 10 mM HEPES, pH 7.4, 2 mM MgCl₂, 10 mM KCI, 1 mM PMSF, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 mM sodium orthovanadate, 10 mM β-glycerophosphate, 50 mM sodium fluoride, and 0.5 mM dithiothreitol. After centrifugation (14,000 g for 45 min), the supernatant containing the cytosolic fraction was dispensed. The pellet was reconstituted with lysis buffer containing 0.1% Triton X-100. After new centrifugation (14,000 g for 45 min), the supernatant containing the solubilized membrane fraction was collected. The protein concentration was determined using the Bio-Rad protein assay kit (Bio-Rad, São Paulo, SP, Brazil) and the samples were stored at −70°C until analysis.

Western blot analysis. Equivalent amounts of proteins (50 μg) were mixed in buffer (200 mM Tris, 10% glycerol, 2% SDS, 2.75 mM β-mercaptoethanol, and 0.04% bromphenol blue) and boiled for 5 min. Proteins were resolved in 10% SDS gel by electrophoresis. After being transferred to a polyvinylidene fluoride membrane (GE Healthcare, São Paulo, SP, Brazil), the blots were blocked with 5% fat-free dry milk-TBS buffer (tris-buffered saline) overnight at 4°C and then washed with tris-buffered saline and 5% Tween-20 (TBST). The membranes were incubated overnight at 4°C with 1:500 dilution of goat anti-ANKTM1 (TRPA1) polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA). The negative control was carried out by the replacement of the primary antibody by nonimmune goat serum (Santa Cruz Biotechnology), in equivalent concentration. Blots were washed four times with TBST for 5 min followed by incubation with adjusted peroxidase-coupled secondary antibody (1:7,500; Promega, Madison, WI) for 1 h. The transferred proteins were visualized with an enhanced chemiluminescence detection kit according to the manufacturer’s instructions (GE Healthcare). Band density measurements were made using NIH ImageJ 1.36b imaging software.

Organ bath studies. Bladders of sham-operated and SCI animals were carefully removed 2, 7, 14, and 28 days after surgery and separated from connective tissue and adherent fat. Bladder strips, ~10-mm long by 4-mm wide (4 strips for each bladder), were placed in a Petri dish containing Krebs-Henseleit solution (composition in mM: 119.0 NaCl, 4.7 KCl, 1.5 MgSO₄, 2.5 CaCl₂, 25.0 NaHCO₃, 1.2 KH₂PO₄, and 11.0 glucose, pH 7.4). Afterward, bladder strips were then carefully mounted in 5-ml organ baths continuously aerated with 95% O₂−5% CO₂ and maintained at 37°C. Preparations were initially submitted to a basal tension of 1 g, followed by a stabilization period of 60 min. During this period, the Krebs solution was changed every 15 min and the basal tension adjusted to 1 g in the first 10 min. Contractile tension changes were measured by means of force displacement transducers (TRI-201; Letica Scientific Instruments, Barcelona, Spain) and were recorded on a polygraph (Letica Scientific Instruments).

Following the stabilization period, the bladder preparations were contracted following the order: cinnamaldehyde (300 μM) or capsaicin (0.1 μM) and carbacol (0.1 μM). After reaching the point of stability of the tonic contractile response for each substance, the Krebs solution was changed four times followed by a new stabilization period of at least 15 min between exposures. The concentration of each drug was selected on the basis of previous studies (2, 9).

In another set of experiments, the effect of TRPA1 antagonism in vitro was tested on the TRPA1-mediated contractile response. Bladders from sham-operated and SCI animals were removed 14 days after surgery and placed in the organ bath as described above. After the stabilization period, the preparations were incubated for 20 min with HC-030031 (TRPA1 antagonist: 10, 30, 60, or 100 μM) followed by exposure to the cinnamaldehyde (300 μM).

Moreover, the effect of TRPA1 downregulation in vivo by means of TRPA1 antisense oligodeoxynucleotide (AS-ODN) treatment was assessed on the spontaneous phasic activity of the bladder and the cinnamaldehyde-induced bladder contractile response. For this purpose, bladders from SCI and sham-operated animals treated with TRPA1 AS-ODN or MM-ODN for 7 days were removed 14 days after surgery and placed in the organ bath as described above. After the stabilization period, the bladder preparations were exposed to the cinnamaldehyde (300 μM). The amplitude of the spontaneous phasic activity of the bladder was measured before addition of cinnamaldehyde.

The results obtained in all experiments were expressed as g of tension.

Cystometric parameters. The urodynamic studies were carried out on day 28 after SCI, a time point that all animals showed spontaneous micturition restoration. Under intraperitoneal urethane anesthesia (0.9 g/kg for SCI and 1.2 g/kg for sham-operated animals), a PE-60 polyethylene catheter (Clay Adams, Parsippany, NJ) was inserted via a midline abdominal incision into the bladder through the bladder dome. The intravesical catheter was connected via a three-way stopcock to a pressure transducer (ADInstruments, Castle Hill, Australia) and to a microinfusion pump (Insight Equipamentos Científicos, São Paulo, Brazil) to record intravesical pressure and to infuse saline into the bladder, respectively. Intravesical pressure was recorded continuously using data-acquisition software (PowerLab 8/30; ADInstruments). After catheter implantation, rats were left untouched for the 30 min for bladder stabilization. After this period, animals received a continuous infusion of saline (0.9% NaCl; 37°C) at a rate of 0.1 ml/min.

We assessed the micturition pressure (MP; maximum bladder pressure during micturition), basal pressure (BP; the lowest bladder pressure between micturitions), threshold pressure (TP; bladder pressure immediately before micturition), and the intercontraction interval (ICI). The mean amplitude and number of nonvoiding contractions (NVCs) were also measured. NVCs were defined as rhythmic intravesical pressure increases greater than 5 mmHg from baseline pressure without release of fluid from the urethra.

Saline voided from urethral meatus was collected and the voided volume (VV) was measured. To measure residual volume, saline infusion was stopped at the beginning of the voiding contraction, and the residual volume (RV) was measured by withdrawing saline through the intravesical catheter and then manually expressing the remaining intravesical contents by exerting pressure on the bladder abdominal wall. The bladder capacity (BC) was calculated as VV plus RV. The voiding efficiency (VE) was estimated as a percentage using the following equation: VE = [(VV/BC) × 100].

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The cystometric parameters were calculated from voiding cycles obtained over 45 min. When investigating the effects of TRPA1 antagonist HC-030031, reproducible micturition cycles were recorded before (used as baseline values) and after administration of the drug. The SCI animals received HC-030031 (30 mg/kg iv caudal) or vehicle (8% DMSO + 2% Tween 80 iv caudal). In another set of experiments, the cystometric parameters were evaluated in SCI animals that received TRPA1 AS-ODN or MM-ODN treatment for 14 days.

Drugs and solutions. The following drugs were used: cinnamaldehyde, carbachol, pentobarbital sodium, urethane (Sigma, St. Louis, MO), oxytetracycline hydrochloride (Terramicin; Pfizer, São Paulo, Brazil), ketamine (Francotar) and xylazine (Virbac, São Paulo, Brazil), sodium heparin (Liquemine; Roche, Basel, Switzerland), and hematoxylin and eosin (Merck, Darmstadt, Germany). Cinnamaldehyde solution was prepared in ethanol and Tween 80 (final bath concentrations of both reagents did not exceed 0.003%). Carbachol solution was prepared in deionized water. HC-030031 (purity ≥98%, HPLC) was synthesized by Dr. Paulo C. Leal as described by the World Intellectual Property Organization (22) and solubilized in DMSO and Tween 80. For in vitro and in vivo experiments, the final concentrations of DMSO did not exceed 0.6 and 8%, respectively. The vehicles used had no pharmacological effects either on the tonus of preparations or the agonist-induced contractions or cystometric parameters.

Statistical analysis. The statistical significance between groups was assessed by paired or unpaired Student’s t-test, Mann-Whitney U-test, or one-way ANOVA followed by the Student-Newman-Keuls test. P values <0.05 (P < 0.05) were considered to be indicative of significance (Prism 4; GraphPad Software, San Diego, CA). Data are presented as means ± SD.

RESULTS

Pathophysiological alterations after SCI. BBB scores observed in our experiments were quite similar to those previously reported by Ref. 34, with a normal locomotor activity (score 21) for sham-operated animals and score of 0 for both hindlimbs on day 2 for SCI animals, which was gradually increased to a final score of 3.2 ± 1.2 (***P < 0.001) 4 wk post-SCI, characterizing severe paraplegia (see Supplemental Fig. S1; the online version of this article contains supplemental data). The SCI animals that had a score above 4 were not used in the subsequent experiments.

The histological analysis of the bladders from SCI animals revealed the presence of fibrin, vascular alterations, edema, and reduction in the number of urothelium layers, compared with bladders from sham-operated animals (Fig. 1, A–C). On the second day after SCI, the damage observed in urothelium was severe, with a widespread loss of stratification. At this time point, a marked neutrophil infiltration to the suburothelial...
tissue was observed (Fig. 1B). To validate the histological observations, bladders were processed for MPO analysis, which showed an increase of ~70-fold in the enzyme activity in SCI bladders as compared to sham bladders (0.05 ± 0.01 vs. ± 3.3 ± 0.7 DO/mg tissue; **P < 0.001), an effect that returned to basal values in the subsequent periods (Fig. 1D). In bladder tissues collected after the second day following SCI, urothelial cells were recovered and only discrete and sparse stratification perturbations were noted (Fig. 1C). Nonetheless, SCI caused a long-lasting increase in bladder wet weight [day 2–sham: 26.0 ± 0.4, SCI: 43.3 ± 5.9 (1.7-fold increase); day 7–sham: 27.4 ± 3.7, SCI: 81.1 ± 21.7 (3-fold increase); day 14–sham: 27.5 ± 4.9, SCI: 81.3 ± 21.2 (3-fold increase); day 28–sham: 22.0 ± 10.9, SCI: 87.3 ± 30.6 mg (3.5-fold increase); **P < 0.001], indicating tissue hypertrophy (Fig. 1E). Taken together, these results suggest that SCI induces an acute inflammatory response and a prolonged structural change in bladder.

Effect of SCI on TRPA1 mRNA levels. At different time points after surgery, bladders, DRG, and spinal cord tissues from naive, sham-operated, and SCI animals were removed and the TRPA1 mRNA levels were measured by real-time reverse-transcription PCR. Interestingly, on day 7 after the surgical procedure, the TRPA1 mRNA expression was increased in both bladder and DRG neurons by 9.2-fold [sham: 0.6 ± 0.5; SCI: 5.5 ± 2.4 relative quantification (RQ); **P < 0.001] and 2.2-fold [sham: 2.1 ± 1.5; SCI: 4.7 ± 1.2 RQ; *P < 0.05], respectively (Fig. 2, A and B). However, SCI did not result in any significant alteration on TRPA1 mRNA level in spinal cord (Fig. 2C).

Effect of SCI on TRPA1 protein levels. Figures 3 and 4 show the TRPA1 expression in bladder, DRG, and spinal cord tissues obtained from naive, sham-operated, and SCI animals in different time points after surgery. The immunohistochemistry analysis shows that TRPA1 was markedly upregulated in bladder on days 7, 14, and 28 after SCI (4.3-, 2-, and 3.3-fold increase, respectively) [day 7–sham: 10.4 ± 3.5, SCI: 45.3 ± 22.2; day 14–sham: 21.1 ± 10.5, SCI: 61.0 ± 19.2; day 28–sham: 19.9 ± 10.9, SCI: 65.9 ± 15.7 arbitrary units; ***P < 0.001; Fig. 3, A–C]. Accordingly, the Western blot analysis also shows TRPA1 upregulation in the same periods after SCI [day 7–sham: 15.7 ± 1.0, SCI: 34.7 ± 1.2; day 14–sham: 12.7 ± 6.7, SCI: 31.0 ± 7.7; day 28–sham: 15.2 ± 1.0, SCI: 28.4 ± 6.4 arbitrary units (2.2-, 2.4-, and 1.8-fold increase, respectively; *P < 0.05; Fig. 3D)]. Moreover, SCI induced the TRPA1 upregulation in DRG on days 7 and 14 after surgery [day 7–sham: 0.7 ± 1.1, SCI: 32.9 ± 16.8; day 14–sham: 0.1 ± 0.1, SCI: 5.9 ± 4.4 arbitrary units (48- and 52-fold increase, respectively; P < 0.05, ***P < 0.001)], compared with the respective sham group (Fig. 4, A–C). Similar to the data observed in the TRPA1 mRNA levels, there was no significant alteration in the protein expression in the spinal cord tissue similarly to the data observed in the TRPA1 mRNA levels (Fig. 4, D–F). No staining or band was observed in control experiments (Supplemental Fig. S2 and Fig. 3D).

Bladder activity after SCI. At days 14 and 28 after surgery, SCI bladder strip contractile responses to cinnamaldehyde (300 μM) were markedly increased compared with the sham bladder strips [day 14–sham: 0.7 ± 0.1, SCI: 1.5 ± 0.4 (2.1-fold increase; *P < 0.05); day 28–sham: 0.9 ± 0.4, SCI: 1.8 ± 0.6 g (2-fold increase; ***P < 0.01); Fig. 5A]. Interestingly, the bladder contractile response induced by capsaicin (0.1 μM) was also significantly higher in SCI group at these same periods [day 14–sham: 0.8 ± 0.3, SCI: 2.4 ± 1.2 (3-fold increase); day 28–sham: 1.0 ± 0.6, SCI: 2.8 ± 0.9 g (2.8-fold increase); *P < 0.05; Fig. 5B]. Concerning the effect of carbobalchol (0.1 μM), SCI significantly potentiated the contractile response to this agent in all periods assessed [day 2–sham: 0.9 ± 0.3, SCI: 1.7 ± 0.6; day 7–sham: 1.0 ± 0.3, SCI: 2.8 ± 0.9 g; day 14–sham: 1.1 ± 0.3, SCI: 2.1 ± 0.4; day 28–sham: 1.0 ± 0.3, SCI: 3.1 ± 1.4 g (1.9-, 2.8-, and 3.1-fold increase, respectively; ***P < 0.001); Fig. 5C].

At 14 days after surgery, the preincubation of the bladder preparations with the TRPA1 receptor antagonist HC-030031 (10, 30, 60, or 100 μM) inhibited, in a concentration-dependent manner, the cinnamaldehyde (300 μM)-mediated contractile response [sham–control: 1.0 ± 0.3, 60 μM: 0.4 ± 0.4, 100 μM: 0.01 ± 0.02; SCI–control: 1.5 ± 0.2, 10 μM: 0.9 ± 0.2, 30 μM: 0.7 ± 0.1, 60 μM: 0.6 ± 0.1, 100 μM: 0.1 ± 0.3 g; ***P < 0.01]. The estimated mean IC50 values were: 46.3 (23.7–68.9) for sham group and 31.2 (9.4–53.0) μM for SCI group (Fig. 6A).

To further evaluate the role of TRPA1 in bladder contractility, sham-operated and SCI animals were treated intrathecally with AS-ODN targeting TRPA1 for 7 consecutive days and the bladders were collected on day 14 for in vitro analysis. Comparatively to the sham group, the SCI group showed an important change in the spontaneous phasic activity of the bladder characterized by enhancement ~4.3-fold in its amplitude [sham: 0.3 ± 0.2, SCI: 1.3 ± 0.9 g; ###P < 0.01; Fig. 6B]. This effect was significantly reduced in the SCI group treated with TRPA1 AS-ODN group compared with the SCI group treated with TRPA1 MM-ODN (53.5 ± 10.4%, from 1.3 ± 0.9 to 0.6 ± 0.6 g; *P < 0.05; Fig. 6C).
Of great relevance, the treatment of SCI animals with TRPA1 AS-ODN reduced the cinnamaldehyde-induced contractile response (53.4 ± 7.2%, from 2.4 ± 1.0 to 1.2 ± 0.6 g; **P < 0.01) reestablishing the similar values displayed by the sham group treated with TRPA1 MM-ODN (1.3 ± 0.1 g; Fig. 6C). Moreover, the TRPA1 AS-ODN treatment also reduced the cinnamaldehyde-induced contractile response in sham-operated animals (52.4 ± 7.3%, from 1.2 ± 0.3 to 0.5 ± 0.2 g; **P < 0.01; Fig. 6C). On the other hand, the carbachol-induced contractile response was not significantly altered by TRPA1 AS-ODN treatment (Fig. 6D).

Cystometry. We investigated the urine voiding behavior by measuring the intravesical pressure and volume in sham-operated and SCI animals. During the bladder-filling phase, SCI animals showed more NVCs than sham-operated animals (sham: 1.0 ± 0.7; SCI: 13.2 ± 6.5; ***P < 0.01; Table 1 and Fig. 7, A and B). Additionally, sham-operated animals showed a large number of voiding contractions in this phase (Fig. 7A).

Moreover, SCI animals displayed marked alterations such as reduction in ICI (78.8 ± 1.6%, sham: 3.5 ± 1.9; SCI: 0.7 ± 0.2 min; ***P < 0.001), in VV (84.9 ± 2.2%, sham: 0.5 ± 0.3, SCI: 0.07 ± 0.02 ml; ***P < 0.01), and in VE (97.1 ± 0.5%, sham: 77.7 ± 11.6, SCI: 2.1 ± 1.0%; ***P < 0.001) and addition to an increase in BP (186.5 ± 25.7%, sham: 5.3 ± 1.8, SCI: 15.2 ± 3.6 mmHg; **P < 0.01), in TP (70.5 ± 18.2%, sham: 8.9 ± 2.0, SCI: 15.1 ± 5.2 mmHg; *P < 0.05), and in BC (435.0 ± 50.7%, sham: 0.6 ± 0.4, SCI: 3.5 ± 0.9 ml; ***P < 0.001) compared with sham-operated animals (Table 1). No alterations in the urodynamic parameters were observed between naive and sham groups (data not shown).

The intrathecal treatment of SCI animals with the TRPA1 AS-ODN for 14 days reduced only the number of NVCs (52.1 ± 15.6%, from 13.8 ± 7.8 to 6.6 ± 4.8; tP < 0.05; Table 1), while the acute systemic treatment with the TRPA1 receptor antagonist HC-030031 (30 mg/kg iv) reduced the number (67.0 ± 8.5%, from 15.4 ± 3.5 to 6.5 ± 3.6; *P < 0.05) and the amplitude (42.1 ± 15.0%, from 21.5 ± 4.8 to 12.4 ± 7.2 mmHg; *P < 0.05) of NVCs (Fig. 7, B–E). No changes were observed in the ICI, BP, TP, MP, VV, BC, and VE after administration of TRPA1 AS-ODN (Table 1) or HC-030031 (data not shown).

**TRPA1 expression after TRPA1 AS-ODN treatment.** The treatment with TRPA1 AS-ODN for 14 days significantly reduced the expression of TRPA1 protein in the whole bladder (45.1 ± 4.3%, from 52.8 ± 13.8 to 29.8 ± 14.3 arbitrary units; ***P < 0.001), in DRG L6-S1 (66.1 ± 15.0%, from 17.2 ± 14.3 to 7.8 ± 7.2 arbitrary units; *P < 0.05), and in corresponding segment of spinal cord (67.4 ± 7.0%, from 5.8 ± 2.0 to 1.3 ± 1.5 arbitrary units; *P < 0.05) compared with TRPA1 MM-ODN treatment (Fig. 8, A–J). The TRPA1 expression in bladder of SCI animals was significantly higher in urothelial cells (74.6 ± 4.2%) than in detrusor muscle (6.9 ± 1.4 arbitrary units; #P < 0.01; Fig. 8B). Of interest, the TRPA1 AS-ODN treatment reduced only the TRPA1 expression in detrusor (57.6 ± 4.7%, from 3.9 ± 1.5 to 1.7 ± 1.1 arbitrary units; ***P < 0.001; Fig. 8, B–D).

**DISCUSSION**

One of the major findings that emerge from the present study is the pronounced upregulation of TRPA1 protein and mRNA...
levels in both bladder and DRG (L6-S1) neurons from injured animals. Of great relevance, the pharmacological blockade or the downregulation of TRPA1 significantly improved the OAB in SCI rats evidenced by the reduction in the number and amplitude of NVCs and in the spontaneous phasic activity of bladder.

SCI produces severe deficits within the lower urinary tract. The majority of these deficits result from disruption of su-

Fig. 4. Immunohistochemistry analysis of TRPA1 expression in DRG (L6-S1; A) and corresponding segment of the spinal cord (D) of naive, sham-operated, and SCI rats assessed 2, 7, 14, and 28 days after surgery. Representative images of TRPA1 immunostaining in DRG and spinal cord on day 7 after surgery of sham (B and E, respectively) and SCI (C and F, respectively) groups (scale bar = 100 μm). Results are represented as arbitrary units. Each column represents the mean and vertical lines show the SD of 3 animals. *P < 0.05, ***P < 0.001, compared with the respective sham group (1-way ANOVA followed by Student-Newman-Keuls test).

Fig. 5. Contractile response induced by cinnamaldehyde (300 μM; A), capsaicin (0.1 μM; B), or carbachol (0.1 μM; C) in the isolated bladder of sham-operated and SCI animals assessed 2, 7, 14, and 28 days after surgery. Results are expressed as g of tension. Each column represents the mean and vertical lines indicate the SD of 4–6 experiments. *P < 0.05, **P < 0.01, and ***P < 0.001 compared with the respective sham group (1-way ANOVA followed by Student-Newman-Keuls test).
praspinal input, afferent input to the spinal cord, and from the intraspinal circuit reorganization in response to injury (35, 36). Among the functional alterations observed after SCI is the bladder overactivity, a syndrome which origin is believed to be associated with C fibers properties alterations. In this pathology, C fibers become mechanosensitive and responsive to bladder filling, mediating the spinal micturition reflex (38, 41).

Recently, TRPA1 receptor expression has been described in rodent and human bladders, in both C fibers and urothelial cells, where it is suggested to act as mechanosensor and nociceptor in either physiological or pathological states (14, 31). Moreover, it has been recently demonstrated that TRPA1 expression is upregulated in the bladder mucosa of patients with OAB caused by bladder outlet obstruction (13). Our present data reinforce this hypothesis by demonstrating that TRPA1 seems not to be involved in the modulation of the bladder overactivity, a syndrome which origin is believed to be associated with bladder outlet obstruction (13). Our present data reinforce this hypothesis by demonstrating that TRPA1 expression in DRG peaked at day 7, decreasing thereafter, while in the bladder it was long-lasting. These data suggest that the SCI-induced OAB seems to be associated with the TRPA1 upregulation, especially in afferent terminals and urothelial cells of the bladder.

Voiding reflex comprises an afferent limb projection to the spinal cord, a spinobulbospinal limb organized within the spinal cord and an efferent limb conveyed by parasympathetic nerves to the bladder. Following SCI, this reflex is abolished and, over time, replaced by a spinal micturition reflex organized within the sacral spinal cord (28). Our results suggest that TRPA1 seems not to be involved in the modulation of the spinal reflex micturition after SCI, because TRPA1 upregulation was observed in afferent neurons in DRG (L6-S1), but not in the corresponding portion of spinal cord. Nevertheless, changes in TRPA1 expression in spinal central terminals may not be completely excluded, since the expression of this receptor in specific regions of L6-S1 spinal segment that control the micturition should be analyzed to clarify this point.

Table 1. Changes in urodynamic parameters induced by SCI and effect of intrathecal treatment with TRPA1 MM-ODN (0.5 μmol/μl) or AS-ODN (0.5 μmol/μl) during 14 days in SCI rats

<table>
<thead>
<tr>
<th></th>
<th>Sham (n = 6–8)</th>
<th>SCI (n = 6–8)</th>
<th>MM-ODN (n = 6)</th>
<th>AS-ODN (n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean amplitude of NVCs, mmHg</td>
<td>25.1 ± 6.6</td>
<td>21.2 ± 3.2</td>
<td>20.9 ± 3.9</td>
<td>18.0 ± 10.4</td>
</tr>
<tr>
<td>Number of NVCs</td>
<td>1.0 ± 0.7</td>
<td>13.2 ± 6.5†</td>
<td>13.8 ± 7.8</td>
<td>6.6 ± 4.8§</td>
</tr>
<tr>
<td>Basal pressure, mmHg</td>
<td>5.3 ± 1.8</td>
<td>15.2 ± 3.6†</td>
<td>11.8 ± 5.0</td>
<td>13.9 ± 5.3</td>
</tr>
<tr>
<td>Pressure threshold, mmHg</td>
<td>8.9 ± 2.0</td>
<td>15.1 ± 5.2*</td>
<td>15.0 ± 7.4</td>
<td>14.5 ± 5.0</td>
</tr>
<tr>
<td>Maximum voiding pressure, mmHg</td>
<td>33.5 ± 13.8</td>
<td>28.1 ± 5.7</td>
<td>27.5 ± 4.9</td>
<td>28.6 ± 6.3</td>
</tr>
<tr>
<td>Intercontraction interval, min</td>
<td>3.5 ± 1.9</td>
<td>0.7 ± 0.2†</td>
<td>0.9 ± 0.5</td>
<td>1.5 ± 1.8</td>
</tr>
<tr>
<td>Voided volume, ml</td>
<td>0.5 ± 0.3</td>
<td>0.07 ± 0.02‡</td>
<td>0.1 ± 0.1</td>
<td>0.2 ± 0.3</td>
</tr>
<tr>
<td>Bladder capacity, ml</td>
<td>0.6 ± 0.4</td>
<td>3.3 ± 0.9‡</td>
<td>3.6 ± 1.0</td>
<td>4.2 ± 1.8</td>
</tr>
<tr>
<td>Voiding efficiency, %</td>
<td>77.7 ± 11.6</td>
<td>2.1 ± 10‡</td>
<td>5.4 ± 5.5</td>
<td>9.7 ± 18.2</td>
</tr>
</tbody>
</table>

Values are means ± SD. SCI, spinal cord injury; TRPA1, transient receptor potential ankyrin 1; MM-ODN, mismatch oligodeoxynucleotide; AS-ODN, antisense ODN; NVC, nonvoiding contractions. *P < 0.05, †P < 0.01, ‡P < 0.001 compared with sham group (Student’s unpaired t-test) and §P < 0.05 compared with MM-ODN-treated group (Student’s unpaired t-test).
Structural change in bladder is a common consequence of SCI. It has been reported that the increased work load on the bladder wall due to bladder-sphincter dyssynergia and urinary retention following SCI results in detrusor muscle hypertrophy (19). In fact, our results revealed a marked increase in weight bladder of SCI animals, which may result from hypertrophy in response to excessive bladder stretch. However, weight bladder increase could also be caused partially by the edema produced during bladder inflammatory process after SCI.

Recent studies showed that oxidative stress products generated at the inflammatory site, namely H2O2, lipid peroxidation products such as 4-hydroxynonenal, and 15-deoxy-Δ12,14-prostaglandin J2, all are capable of activating TRPA1 and in turn inducing robust calcium influx in cultures of TRPA1-transfected cells and of DRG and nodose ganglion neurons (1, 32). Of interest, the modulation of ion channels in sensory fibers and urothelial cells by mechanical stimuli and cytokines inflammatory can lead to voiding disorders (26). In fact, intravesical administration in rats of TRPA1 activators or the inflammatory mediator H2S is able to induce OAB in rats by increasing the micturition frequency and reducing the VV (14, 31). In this context, several studies demonstrated the upregulation of muscarinic and TRPV1 receptors in bladders of humans and animals with OAB (3, 8, 16, 18, 29). Corroborating these data, our data showed the TRPA1 upregulation, an effect that can explain, at least in part, the enhancement of cinnamaldehyde-induced contractile response in SCI bladders. Bladder smooth muscle exhibits spontaneous phasic activity during filling phase or when isolated bladder strips are placed under tension (27). An increase in amplitude of the spontaneous phasic activity of bladder was reported in humans and in animals with OAB (17). Our data corroborate and largely extend the literature data by demonstrating that the higher amplitude of spontaneous phasic activity observed in SCI bladders was significantly reduced by the intrathecal treatment with TRPA1 AS-ODN, suggesting that TRPA1 downregulation could result in reduced neurotransmitters and local mediators release, responsible by modulation of spontaneous phasic activity of the bladder. In addition, our results demonstrated that this treatment failed to interfere with the spontaneous phasic activity in bladders of sham-operated animals. Therefore, TRPA1 seems to play an important role in only altered spontaneous phasic activity.

Although TRPA1 does not seem to exert a relevant role in normal spontaneous phasic activity, our results showing that the intrathecal treatment with TRPA1 AS-ODN and the exposure of bladder preparations to HC-030031 reduced the cinnamaldehyde-induced contractile response in sham and SCI bladders, suggesting that this receptor could participate in normal and exacerbated bladder contractile response. In addition, TRPA1 AS-ODN did not change the carbachol-induced contractile response in both experimental groups, suggesting that this treatment selectively reduced TRPA1 expression.

It is important to note that the cinnamaldehyde-induced contractile response in sham and SCI bladders was not completely abolished by TRPA1 AS-ODN treatment. The redu-
tion of TRPA1 protein expression in both spinal cord and DRG neurons further confirmed the efficiency of the TRPA1 AS-ODN treatment, which seems to reach the lumbar/sacral DRGs and consequently reduce TRPA1 protein in sensory neuron terminals. However, TRPA1 expression in urothelium was not decreased by this treatment, which could explain the partial reduction in cinnamaldehyde-induced contractile response. In fact, in early stages of SCI in which the cell loss is pronounced,

Fig. 8. Effect of the intrathecal treatment with TRPA1 MM-ODN (0.5 nmol/µl) or AS-ODN (0.5 nmol/µl) on TRPA1 expression in whole bladder (A), bladder urothelium and detrusor muscle (B), DRG (L6-S1; E), and corresponding segment of the spinal cord (H) of SCI rats assessed 28 days after surgery. Representative images of TRPA1 immunostaining in bladder (C and D), DRG (F and G), and spinal cord (I and J) after TRPA1 MM-ODN or AS-ODN treatment. Image of bladder shows staining in detrusor muscle (arrowhead) and urothelium (arrow; scale bars = 100 µm). Results are represented as arbitrary units for A, E, and H and arbitrary units/area for B. Each column represents the mean and vertical lines show the SD of 4–6 animals. *P < 0.05, **P < 0.01, ***P < 0.001, compared with the TRPA1 expression in MM-ODN-treated group and ##P < 0.01 compared with the TRPA1 expression in bladder detrusor muscle of MM-ODN-treated group (Student’s unpaired t-test).
the cinnamaldehyde-induced contractile response was not altered. Additionally, the removal of urothehial cells decreases by \( \sim 60\% \) the cinnamaldehyde-mediated contractile response in bladders of naive animals (result not shown). Therefore, the urothehium actively contributes to bladder contractile response mediated by TRPA1.

Finally, cystometric evaluation performed after SCI showed a significant increase in the number and amplitude of NVCs during the bladder-filling phase, which was inhibited by acute systemic treatment with HC-030031. Nonetheless, the treatment with TRPA1 AS-ODN reduced only the number of NVCs. The effectiveness difference of the treatments might be explained by extension of the area affected by the treatment, i.e., the systemic administration of HC-030031 reaches the activity of the TRPA1 receptor expressed also in urothehium. On the other hand, none of the treatments significantly improved the volume parameters such as VV, RV, BC, and VE. These findings suggest that TRPA1 seems do not exert effect on the activity of spinal motor neurons responsible by control of external urethral sphincter.

In summary, our in vitro and in vivo data provide strong evidences for an important role played by TRPA1 in OAB caused by SCI in rats. Thus, TRPA1 might constitute an attractive and potential target to develop a new therapy for OAB.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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