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Oxaliplatin elicits mechanical and cold allodynia in rodents via TRPA1 receptor stimulation

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ABSTRACT

Platinum-based anticancer drugs cause neurotoxicity. In particular, oxaliplatin produces early-developing, painful, and cold-exacerbated paresthesias. However, the mechanism underlying these bothersome and dose-limiting adverse effects is unknown. We hypothesized that the transient receptor potential ankyrin 1 (TRPA1), a cation channel activated by oxidative stress and cold temperature, contributes to mechanical and cold hypersensitivity caused by oxaliplatin and cisplatin. Oxaliplatin and cisplatin evoked glutathione-sensitive relaxation, mediated by TRPA1 stimulation and the release of calcitonin gene-related peptide from sensory nerve terminals in isolated guinea pig pulmonary arteries. No calcium response was observed in cultured mouse dorsal root ganglion neurons or in naïve Chinese hamster ovary (CHO) cells exposed to oxaliplatin or cisplatin. However, oxaliplatin, and with lower potency, cisplatin, evoked a glutathione-sensitive calcium response in CHO cells expressing mouse TRPA1. One single administration of oxaliplatin produced mechanical and cold hyperalgesia in rats, an effect selectively abated by the TRPA1 antagonist HC-030031. Oxaliplatin administration caused mechanical and cold allodynia in mice. Both responses were absent in TRPA1-deficient mice. Administration of cisplatin evoked mechanical allodynia, an effect that was reduced in TRPA1-deficient mice. TRPA1 is therefore required for oxaliplatin-evoked mechanical and cold hypersensitivity, and contributes to cisplatin-evoked mechanical allodynia. Channel activation is most likely caused by glutathione-sensitive molecules, including reactive oxygen species and their byproducts, which are generated after tissue exposure to platinum-based drugs from cells surrounding nociceptive nerve terminals.
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1. Introduction

Platinum-based drugs are commonly used for the treatment of various types of cancers. Unfortunately, their clinical benefit is challenged by severe adverse effects to the nervous, auditory, hematologic, and renal systems [34]. The third-generation platinum drug oxaliplatin [4] shows a dramatic reduction in renal toxicity and ototoxicity, but exhibits a unique neurotoxic profile [30]. In addition to a chronic sensory neuropathy typical of other platinum-based drugs [30], oxaliplatin causes an acute syndrome, described as cold-exacerbated paresthesia of the hands, feet, perioral region, and throat, which affects almost all patients, and develops rapidly, persisting for several days [47]. Neurotoxicity by platinum-based drugs, and not tumor progression, is often the cause of treatment discontinuation, dose reduction, or hospitalization [39].

Because attempts to mitigate platinum-based drugs' neurotoxicity with neuroprotective strategies have yielded unsatisfactory results, a more precise understanding of the mechanism(s) responsible for the neurotoxic action of platinum-based drugs is required. Ion channels, and particularly voltage sensitive sodium channels, have been proposed as contributors to the neurotoxic action of platinum-based drugs [46,38]. More recently, attention has been paid to the transient receptor potential (TRP) family of ion

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channels. In rodents, deletion of the TRP vanilloid 1 (TRPV1) channel (which is expressed by the largest subset of nociceptive neurons) worsened cisplatin-induced symptoms [9]. In contrast, TRPV1 seems to contribute to the heat-induced hyperalgesia in cisplatin-treated animals [43], whereas acid-sensing ion channels 3 (ASIC3), and the adenosine triphosphate gated $P2X_3$ channel, seem to contribute to mechanical hyperalgesia [20], and the TRP melastatin 8 (TRPM8) to oxaliplatin-evoked cold allodynia [17].

At least part of the cytotoxic effect of oxaliplatin is mediated by its ability to generate reactive oxygen species (ROS) [26,32], and cisplatin-evoked damage of mouse cochleae is associated with the generation of lipid peroxidation byproducts, including 4-hydroxynonenal [29]. In addition, it has recently been reported that oxaliplatin-induced mechanical hyperalgesia, and heat- and cold-evoked allodynia in rats, is attenuated by antioxidants, including acetyl-L-carnitine, α-lipoic acid, or vitamin C, suggesting that oxidative stress contributes to these painful conditions [22].

The TRP ankyrin 1 (TRPA1), a calcium-permeable cation channel co-expressed with TRPV1 in a subpopulation of nociceptive primary sensory neurons [41,21,37], is activated by pungent ingredients present in an array of spices, including allyl isothiocyanate (mustard, wasabi), cinnamaldehyde (cinnamon), and others [21,37]. Robust evidence has been accumulated indicating that ROS [2,8,40] and various endogenous byproducts deriving from ROS induced peroxidation of plasma membrane phospholipids, including acrolein [7] and 4-hydroxynonenal [45] gate TRPA1, thereby causing pain and neurogenic inflammation. It has been reported [41] and more recently confirmed [24] that TRPA1 is activated by noxious cold temperatures. Thus, because TRPA1 is a sensor of both oxidative stress [8] and cold temperatures [24], we have hypothesized that TRPA1 mediates mechanical and cold hypersensitivity provoked by platinum-based anticancer drugs. Our data show that in rat and mouse models, TRPA1 is a main contributor of mechanical and cold hyperalgesia/allodynia induced by oxaliplatin or cisplatin, thus shedding light on the mechanisms underlying painful sensory neuropathy associated with the use of these drugs.

2. Materials and methods

2.1. Reagents

If not otherwise indicated, all reagents were from Sigma–Aldrich (Milan, Italy). HC-030031 was synthesized as previously described [3]. Olcegepant was kindly donated by Dr. H. Doods (Boehringer-Ingelheim, Inselheim, Germany).

2.2. Animals

All animal experiments were carried out in accordance with the European Union Community Council guidelines and approved by the local ethics committee. Dunkin-Hartley guinea pigs (male, 250 g) (Charles River, Milan, Italy), Sprague–Dawley rats (male, 250 g), C57BL/6 mice (male, 25 g) (Harlan Laboratories, Milan, Italy), wild-type (*Trpa1*$^{+/+}$) or TRPA1-deficient mice (*Trpa1*$^{-/-}$), generated by heterozygous mice on a C57BL/6 background [7], were used. Behavioral experiments were done in a quiet, temperature-controlled room (20°C to 22°C) between 10 am and 4 pm and were performed with an operator blinded to the genotype and the status of drug treatment. Animals were killed with a high dose of intraperitoneal (i.p.) sodium pentobarbital (200 mg/kg).

2.3. Organ bath studies

Pulmonary arteries were excised from guinea pigs and suspended at a resting tension of 1 g in 10-mL organ baths, and the assay was performed as previously described [33]. The tissues were bathed in aerated (95% O_2 and 5% CO_2) Krebs–Henseleit solution containing (in mM): 119 NaCl, 25 $NaHCO_3$, 1.2 KH_2PO_4, 1.5 $MgSO_4$, 2.5 $CaCl_2$, 4.7 KCl, and 11 D-glucose, maintained at 37°C, which contained the neutral endopeptidase inhibitor thiorphan (10 μM) and the muscarinic antagonist atropine (10 μM). After 45 minutes of equilibration, tissues were contracted twice with phenylephrine (1 μM), with a 45-minute washing out period between the first and second administration. Once the contractile response to the second administration of phenylephrine had reached a plateau, oxaliplatin (100 μM), cisplatin (100 μM), mustard oil (MO, 10 μM) or their respective vehicles (0.1%, 0.1%, and 0.01%, dimethyl sulfoxide [DMSO]) and capsaicin (1 μM) or its vehicle (0.01% ethanol) were added and left in contact with the preparation for the entire duration of the experiment. In some experiments, tissues were pretreated with the TRPA1 blocker, HC-030031 (10 μM), the calcitonin gene-related peptide (CGRP) receptor antagonist, olcegepant (BIBN4096BS, 10 μM), the TRPV1 blocker, capsazepine (CPZ; 10 μM), or their respective vehicles (0.1% DMSO for HC and BIBN4096B, 0.1% ethanol for CPZ). In addition, some tissues were pretreated with glutathione (GSH, 1 mM) or its vehicle (isotonic saline), 1 hour prior to the second administration of phenylephrine. Some tissue preparations were desensitized to capsaicin (10 μM for 20 minutes) as previously reported [3]. Motor activity of tissue preparation was recorded isometrically on a force transducer (Harvard Apparatus, Ltd, Kent, United Kingdom). The results were expressed as percentage of relaxation of the maximum contractile response induced by phenylephrine.

2.4. Cell culture and isolation of primary sensory neurons

A tetracycline-regulated system for inducible expression of TRPA1 in Chinese hamster ovary (CHO) cells transfected with the cDNA of the mouse TRPA1 (mTRPA1-CHO) was used, as described previously [41]. Naïve CHO cells were used as controls. The primary cell culture of sensory neurons has been described previously for trigeminal ganglia, and was slightly modified for dorsal root ganglia (DRG) neurons [24]. Lumbosacral (L5-S2) DRGs were bilaterally excised under a dissection microscope. The ganglia were washed in phosphate-buffered saline solution (PBS, Invitrogen, Carlsbad, California, USA) and collected in ice-cold Leibowitz medium (L15, Invitrogen). The ganglia were then transferred into warm (37°C) Dulbecco modified essential medium (DMEM) containing 0.025% collagenase (type IA) and incubated (95% air, 5% CO_2) at 37°C. After 45 minutes, tissue was gently triturated with a fire-polished glass pipette, and the resultant suspension was centrifuged at 1700 rpm for 10 minutes. The pellet was resuspended in DMEM/F-12 (1:1) containing Glutamax (Invitrogen) supplemented with 10% fetal calf serum (Invitrogen) and 100 μg/mL penicillin/streptomycin (Invitrogen). Cells were plated on poly-L-lysine-coated glass coverslips and used after 1 day in culture. In some experiments, isolated DRG neurons were co-cultured with naïve CHO cells. DRG neurons were plated on poly-L-lysine-coated glass coverslips and 1 hour afterward, naïve CHO cells have been plated in the same coverslips. Co-cultures were maintained with the same medium used for DRG neurons and used 1 day afterward.

2.5. Calcium imaging experiments

Cells were incubated with 2 μM Fura-2AM ester for 30 minutes at 37°C. Intracellular calcium ($[Ca^{2+}]_i$) concentration was measured on an Olympus (Tokyo, Japan) CellM system. Fluorescence was measured during excitation at 340 and 380 nm, and after correction for the individual background fluorescence signals, the ratio of the fluorescence at both excitation wavelengths (F_{340}/F_{380}) was monitored. Experiments were performed using the standard Krebs

solution containing (in mM): 150 NaCl, 6 KCl, 1 $MgCl_2$, 1.5 $CaCl_2$, 10 glucose, 10 HEPES and titrated to pH 7.4 with 1 N NaOH. Cells were exposed to oxaliplatin and cisplatin, (both, 10 to 300 μM), MO (100 μM), and platinum dichloro diaminocyclohexane (300 μM) or their respective vehicles (0.3%, 0.1%, and 0.3% DMSO) in the presence of GSH (1 mM) or its vehicle (isotonic saline). To identify neurons in DRG neuron cultures, we applied a Krebs-based solution in which the KCl concentration was increased to 50 mM by iso-osmotic substitution of NaCl.

2.6. Detection of oxaliplatin-GSH adducts by high-performance liquid chromatography–electrospray mass spectrometry (HPLC/ESI-MS)

Oxaliplatin (100 μM) and GSH (1 mM) were incubated in equivolumetric amounts in a water medium at 37°C. Samples were taken at time 0 (preincubation) and after 10 minutes of incubation and analyzed by means of HPLC/ESI-MS. HPLC separations were performed at 25°C using a Hypersil PFP column (150 × 2.1 mm, particle size 3 μm) with a linear gradient. The mobile phase was MeOH/H_2O/HCOOH (0.1% v/v) and the gradient program was 10% to 45% v/v MeOH in 14 minutes. The flow rate was 50 μL/min, and the injection volume was 10 μL. All measurements were repeated twice. ESI source parameters (positive polarity) were: spray voltage 4 kV, capillary temperature 400°C, capillary voltage 18 V, tube lens 110 V, skimmer offset 0 V. The parallel estimation of the variation of intensities of chromatographic peaks corresponding to GSH and oxaliplatin absorption, expressed as percentage areas, was performed by HPLC analysis following previously reported conditions (C_{18} reverse-phase column eluted with a CH_3CN/H_2O gradient containing 0.1% of the ion pairing agent, monitored at 220 nm) [16].

2.7. Superoxide anion production assay

Naïve CHO cells were grown in 6-well plates until 90% to 100% confluence, and starved in serum-free DMEM overnight before treatment. Similarly, cells (including neuronal and nonneuronal cells) isolated from rat DRG ganglia were plated on poly-L-lysine-coated 6-well plates at a density equivalent of 20 ganglia per well, obtaining a 70% to 80% confluence. Cells were then incubated either with oxaliplatin or cisplatin, both 300 μM, or their vehicle (0.3% DMSO), in serum-free DMEM containing cytochrome C (1 mg/mL) for 2 hours at 37°C, in the presence or absence of bovine superoxide dismutase (SOD) (300 U/mL). The supernatants were collected, and the optical density was spectrophotometrically measured at 550 nm. The superoxide anion amount was calculated by using an extinction coefficient of 2.1×10^4/M/cm and expressed as nmol/mg protein/2 hours.

2.8. Oxaliplatin and cisplatin-induced painful neuropathy models

After habituation and baseline measurements of pain sensitivity, animals were randomized into treatment groups. Rats were treated with a single intravenous (i.v.) administration of oxaliplatin (2 mg/kg) or its vehicle (isotonic saline) [23]. C57BL/6, *Trpa1*$^{+/+}$, or *Trpa1*$^{-/-}$ mice were treated with a single i.p. administration of oxaliplatin (3 mg/kg) or its vehicle (isotonic saline) [17]. In another set of experiments, C57BL/6 or *Trpa1*$^{+/+}$ and *Trpa1*$^{-/-}$ mice were treated with repeated doses of cisplatin (2 mg/kg, i.p.) or its vehicle (isotonic saline) three times per week over 5 weeks (cumulative dose of 30 mg/kg) [9]. Starting the third week of treatment with cisplatin, the animals began to lose weight, whereas no weight loss was observed after oxaliplatin treatment. Oxaliplatin and cisplatin were dissolved in isotonic saline, and the volume was adjusted to 1 mL/kg for i.v. and 10 mL/kg for i.p. administration.

2.8.1. Randall-Selitto paw-withdrawal test

Oxaliplatin-induced mechanical hyperalgesia was assessed by measuring the paw-withdrawal thresholds in rats using an Ugo Basile analgesimeter (Ugo Basile, Varese, Italy). Briefly, rats were allowed into individual Plexiglas cages and acclimatized for 10 to 15 minutes, after which the hind paws were exposed to the test stimulus. The paw-withdrawal threshold was determined before (basal level threshold) and after drug administration. The effect of oxaliplatin was tested for 20 days, starting immediately after drug administration, which was counted as day 1. Intragastric (i.g.) HC-030031 (100 mg/kg) or its vehicle (0.5% carboxymethylcellulose) and capsazepine (4 mg/kg, i.p.) or its vehicle (5%, DMSO) were administered at day 2 and 15 after oxaliplatin or vehicle administration. In another experimental setting, rats were treated with an intraplantar injection of capsaicin (20 μg/50 μL/paw) or its vehicle (1% ethanol) and the paw-withdrawal threshold was determined 30 minutes after drug administration [19]. Capsazepine (4 mg/kg, i.p.) or its vehicle (5% DMSO) were administered 10 minutes before capsaicin or vehicle administration. Nociceptive thresholds were determined at 5-minute intervals, and the mean of three readings was defined as the nociceptive threshold. Cutoff pressure was 200 g. Data are expressed as the pressure threshold (g) required to elicit the paw withdrawal.

2.8.2. Von Frey hair test

Oxaliplatin and cisplatin-induced mechanical allodynia was measured in C57/BL6, *Trpa1*$^{+/+}$ or *Trpa1*$^{-/-}$ mice by using the up-and-down paradigm [10]. Mechanical nociceptive threshold was determined before (basal level threshold) and after drug administration. The effect of oxaliplatin was tested for 30 days, starting immediately after drug administration, which was counted as day 1. The effect of cisplatin was tested at 5 weeks after the end of the treatment cycle. Data are expressed as the mean threshold values (g).

2.8.3. Cold stimulation

Cold hyperalgesia induced by oxaliplatin was assessed in rats by using the tail immersion test in a water bath maintained at 10°C, with a cutoff time of 15 seconds. The time taken by the rat to withdraw its tail from the cold water was the nociceptive measure. Three measurements were taken at 5-minute intervals, and were averaged. The baseline of the latency was determined before (basal level) and after drug administration [23]. The effect of oxaliplatin was tested for 8 days, starting immediately after drug administration, which was counted as day 1. HC-030031 (100 mg/kg, i.g.) or its vehicle (0.5% carboxymethylcellulose) was administered at day 2 after the administration of oxaliplatin or its vehicle.

Cold allodynia was assessed in mice by measuring the acute nocifensive responses to the acetone-evoked evaporative cooling as previously described [17]. Briefly, the animal was held by the hand and a droplet (50 μL) of acetone, formed on the flat-tip needle of a syringe, was gently touched to the plantar surface of the hind paw. The mouse was immediately put in a cage with a transparent floor, and the time spent in elevation and licking of the plantar region over a 60-second period was measured. Acetone was applied 3 times at a 10- to 15-minute intervals, and the average of elevation/licking time was calculated. Cold allodynia was measured in mice before (baseline) and for 30 days after drug treatment.

2.9. Real-time polymerase chain reaction (PCR)

For real-time PCR studies, DRGs were isolated from mice treated with oxaliplatin (3 mg/kg, i.p.) or its vehicle before or at different times (6 and 24 hours and 3, 10, and 30 days) after oxaliplatin treatment. Total RNA was isolated from tissues using a tissue homogenizer (ULTRA-TURAX IKA T10 basic, IKA, Staufen,

Germany) and TRIzol Reagent (Invitrogen), according to the manufacturer's instructions. Samples were treated with the RNase-free DNase I (Applied Biosystems, Carlsbad, California, USA) and quantified on a Picodrop spectrophotometer (Picodrop Limited, United Kingdom). Reverse transcription of 500 ng total RNA was carried out using SuperScript III Platinum Two-Step qRT-PCR Kit (Invitrogen) according to the manufacturer's directions. Two sets of primer-probes were designed using the Primer Express Software version 3.0 (Applied Biosystems). The chosen reporter fluorophores for TaqMan MGB probes were VIC for the 28S rRNA internal control and 6-carboxyfluorescein (FAM) for the TRPA1 gene. The 2 sets of primer-probes were as follows: set 1, 28SRNA-FW (forward) 5′-CC GCTAAGGAGTGTGTAACAACTC-3′, 28SRNA-RE (reverse) 5′-CTCCA GCGCCATCCATTT-3′, 28SRNA probe 5′-VIC-CCGAATCAACTAGCCC TG-3′; and set 2, TRPA1-FW (forward) 5′-CAGGATGCTACGGTTT TTTCATTACT-3′, TRPA1-RE (reverse) 5′-GCATGTGTCAATGTTTGGT ACTTCT-3′ and TRPA1 probe 5′-FAM-TCTTAATATGCAAGAAACA CG-3′. The chosen primers and probes were subjected to Basic Local Alignment Search Tool BLAST database searches to find any sequence similarities. Real-time quantitative PCR was performed using StepOnePlus Real-Time PCR System (Applied Biosystems). All samples were run in triplicate in a final volume of 25 μL containing 12.5 μL of 2× TaqMan Gene Expression PCR Master Mix, 300 nM of each primer, 250 nM of each probe, and 4 μL of RT reaction, according to the manufacturer's instructions (Applied Biosystems). Amplification conditions were: 50°C for 2 minutes and 95°C for 10 minutes, followed by 40 cycles of 95°C for 30 seconds and 60°C for 1 minute. The fold change of TRPA1 mRNA levels was calculated relative to the 28S rRNA internal control, using the $2^{-\Delta\Delta CT}$ comparative method.

2.10. Statistical analysis

Data are presented as mean ± SEM. Statistical analyses were performed by the unpaired 2-tailed Student *t* test for comparisons between two groups, the one-way analysis of variance followed by the post hoc Bonferroni test for comparisons of multiple groups, and the chi-square test for comparing two proportions. Values of $P < 0.05$ were considered statistically significant.

3. Results

3.1. Oxaliplatin and cisplatin activate TRPA1 on nociceptive nerve terminals via a glutathione-sensitive mechanism in the guinea pig pulmonary artery

To test the hypothesis that oxaliplatin and cisplatin may activate TRPA1 on sensory nerve terminals, we utilized the isolated guinea pig pulmonary artery assay [33]. In keeping with previous findings [33], the neurogenic relaxation evoked by capsaicin was markedly reduced by pre-exposure of the tissue to an elevated concentration of capsaicin, which renders sensory nerve terminals unresponsive to any stimulus [42]. The CGRP receptor antagonist, olcegepant [13], and the TRPV1 antagonist, capsazepine, but not the TRPA1 selective antagonist, HC-030031 [35], blocked the effect of capsaicin (Fig. 1A and C). The selective TRPA1 agonist, MO, caused relaxation that was inhibited by capsaicin-desensitization, olcegepant, and HC-030031, but not by capsazepine (Fig. 1B), thus suggesting that MO evokes CGRP-mediated relaxation via stimulation of TRPA1-expressing sensory nerve terminals (Fig. 1B). Finally, exposure of guinea pig pulmonary arteries to oxaliplatin (100 μM) or cisplatin (100 μM) caused relaxation that was capsaicin-sensitive and inhibited by olcegepant and HC-030031, but unaffected by capsazepine (Fig. 1C to E). We also found that the ROS and the reactive aldehyde scavenger GSH [1,15] left relaxation evoked by capsaicin or MO unchanged (Fig. 1A and B), but markedly inhibited responses to oxaliplatin and cisplatin (Fig. 1D and E). Thus, in this bioassay platinum-based drugs activate the TRPA1 channel, expressed on capsaicin-sensitive nociceptive nerve terminals, through a GSH-sensitive mechanism.

3.2. Oxaliplatin and cisplatin activate TRPA1 by an indirect mechanism mediated by oxidative stress and release superoxide anion

To test the hypothesis whether platinum drugs directly activate TRPA1, we studied the calcium response to oxaliplatin and cisplatin in cultured mouse DRG neurons, which express TRPA1, and in CHO cells transfected with the cDNA of the mouse TRPA1 (mTRPA1-CHO). Neither oxaliplatin nor cisplatin (both 300 μM) caused any calcium response in mouse DRG neurons (Fig. 2A). In contrast, oxaliplatin evoked a calcium response in mTRPA1-CHO cells (Fig. 2B and D). The number of responding cells increased with the oxaliplatin concentration (10 to 300 μM) (Fig. 2D). The effect of oxaliplatin (300 μM) was almost entirely abated in the presence of GSH (Fig. 2B and D). Cisplatin was a weaker agonist than oxaliplatin, causing a calcium response in a significant number (approximately 40%) of mTRPA1-CHO cells only at the 300 μM concentration (Fig. 2C and D). The response to cisplatin was also abated by GSH (Fig. 2C and D). Neither oxaliplatin nor cisplatin evoked any response in untransfected cells (Fig. 2B and C). We also investigated the effect of oxaliplatin or cisplatin in co-cultures of DRG neurons, obtained from wild-type mice, and naïve CHO cells. Under these conditions, both oxaliplatin (300 μM) and cisplatin (300 μM) caused a calcium response in a small but significant proportion of cells, which also responded to MO. Cells responding to oxaliplatin were 13% (n = 16) of the total number of cells responding to MO (n = 123). Cells responding to cisplatin were 11% (n = 11) of the total number of cells responding to MO (n = 98). The effect induced by both oxaliplatin and cisplatin was 39% ± 7% and 33% ± 7% of that induced by MO, respectively. In the presence of GSH (1 mM) of 70 cells responding to MO, none responded to oxaliplatin. Similarly, no response to oxaliplatin was seen in co-cultures with neurons taken from TRPA1-deficient mice (as expected, under these circumstances, no response to MO was reported). Similar results were obtained with cisplatin. In the presence of GSH, no response to cisplatin was observed in the cells that responded to MO (n = 87). No response to cisplatin or MO was detected in co-cultures with neurons taken from TRPA1-deficient mice.

Next we investigated the mechanism by which GSH blocked the oxaliplatin effect. To this purpose, oxaliplatin (100 μM) and GSH (1 mM) were incubated for a period of time (10 minutes) that exceeded the amount of time required to fully evoke the calcium response by oxaliplatin in mTRPA1-CHO cells. As reported previously [16], prolonged incubation periods (hours) with GSH may reduce the concentration of the intact oxaliplatin. However, HPLC/ESI-MS analysis showed that 10 minutes of incubation with GSH was not sufficient to reduce oxaliplatin concentration (Supplementary Fig. 1). This indicates that inhibition of oxaliplatin-evoked calcium response was not due to a direct inactivation of the chemotherapeutic agent by GSH. Therefore, we propose that platinum-based drugs do not directly activate TRPA1, but rather channel stimulation occurs indirectly via the generation of reactive GSH-sensitive chemical species. The potentially reactive oxaliplatin metabolite, platinum dichloro diaminocyclohexane (300 μM), failed to evoke any calcium response in mTRPA1-CHO (Supplementary Fig. 2).

The level of superoxide anion in the supernatant of naïve CHO cells after exposure to oxaliplatin (300 μM) (6.89 ± 0.93 nmol/mg protein/2 hours, n = 6) was significantly higher ($P < 0.02$) than the level measured after exposure to the vehicle (4.02 ± 0.59 nmol/mg protein/2 hours, n = 8). Similarly, cisplatin (300 μM) increased superoxide anion level (5.95 ± 0.49 nmol/mg protein/2 hours,

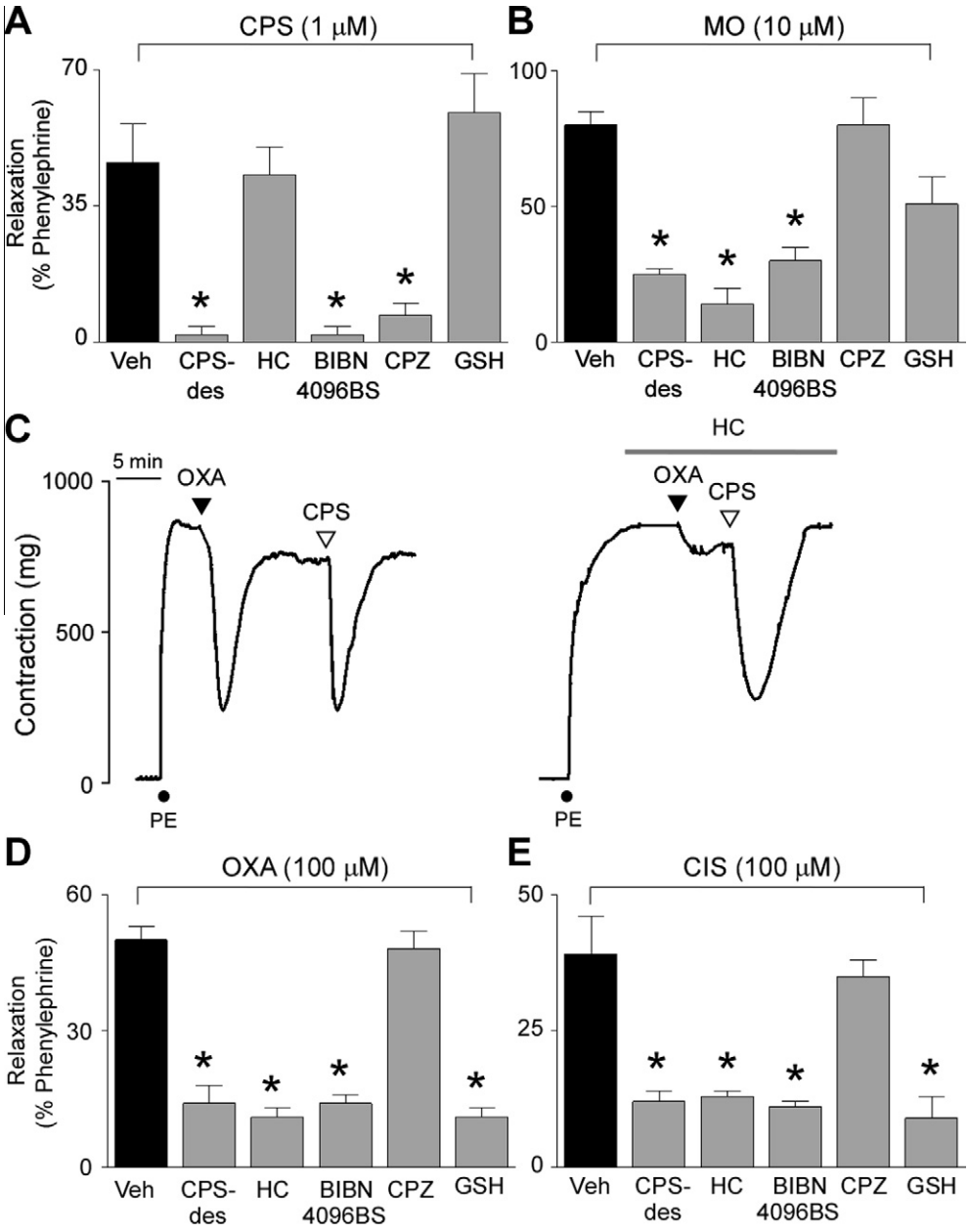

Fig. 1. Oxaliplatin and cisplatin gate TRPA1 channel and induce neurogenic relaxation in guinea pig pulmonary artery via a glutathione (GSH)-dependent mechanism. (A and C) Capsaicin (▽: CPS; 1 μM) relaxes guinea pig isolated pulmonary artery precontracted with phenylephrine (●: PE; 1 μM) via CGRP release from sensory nerves. (A) The relaxation evoked by CPS is abolished by capsaicin desensitization (CPS-des; 20 μM for 20 minutes), by pretreatment with the CGRP receptor antagonist, olcegepant (BIBN4096BS; 10 μM), and the TRPV1 antagonist, capsazepine (CPZ; 10 μM), but was unaffected by the TRPA1 selective antagonist, HC-030031 (HC; 10 μM) and GSH (1 mM). (B) The selective TRPA1 agonist, mustard oil (MO; 10 μM), evokes a similar neurogenic relaxation that is abated by CPS-des, BIBN4096BS, and HC, but is unaffected by CPZ or GSH pretreatment. (C) Representative traces and (D) pooled data of the vasorelaxant effect induced by oxaliplatin (▼: OXA; 100 μM) and (E) cisplatin (CIS; 100 μM) in precontracted pulmonary artery. Relaxation is abated by CPS-des, BIBN4096BS, and HC, but not by CPZ. Pretreatment with GSH completely prevents relaxation induced by OXA and CIS. Veh is the combination of vehicles of the various antagonists. Values are mean ± SEM of n ⩾ 4 experiments. *$P < 0.05$ vs. Veh; one-way ANOVA and Bonferroni test.

n = 5, $P < 0.05$) as compared to the level measured after exposure to the vehicle (4.02 ± 0.61 nmol/mg protein/2 hour, n = 5). In the supernatant of cultured DRG cells, superoxide anion levels were under the sensitivity of the method after exposure to either oxaliplatin and cisplatin or their vehicles (data not shown).

3.3. Oxaliplatin-evoked mechanical and cold hyperalgesia in rats are mediated by TRPA1

Next we investigated whether TRPA1 could be involved in models of mechanical and cold hypersensitivity in rats and mice. In agreement with data reported earlier [23], we found that i.v. administration of oxaliplatin (2 mg/kg) produced an early and remarkable reduction in mechanical paw-withdrawal threshold in rats. The reduction (approximately 60% from baseline), already visible at day 1, remained constant until day 8, then declined progressively to plateau again around day 15 (Fig. 3A). In another set of experiments, the administration of the TRPA1 selective antagonist HC-030031 (100 mg/kg, i.g.) [35] 2 days (Fig. 3B) or 15 days (Fig. 3C) after oxaliplatin administration produced a time-dependent and complete reversal of the mechanical hyperalgesia evoked by oxaliplatin. The effect of HC-030031, in keeping with previous

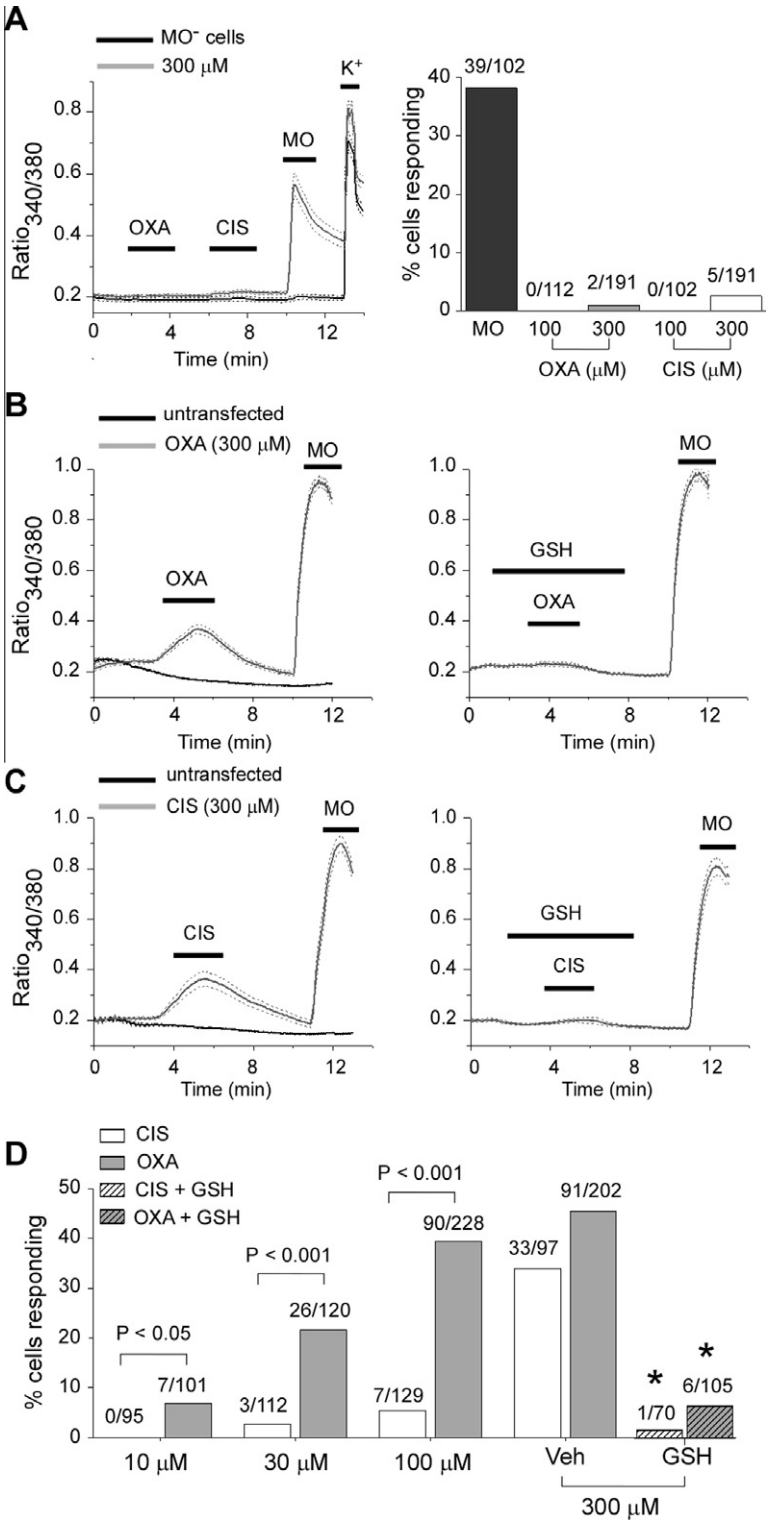

Fig. 2. Oxaliplatin and cisplatin activate TRPA1 by an indirect mechanism mediated by a glutathione (GSH)-sensitive mechanism. (A) Oxaliplatin (OXA; 100 to 300 μM) and cisplatin (CIS; 100 to 300 μM) do not evoke any intracellular calcium ($[Ca^{2+}]_i$) response in mouse dorsal root ganglion (DRG) neurons (identified by the response to 50 mM K^+), which respond to mustard oil (MO; 100 μM) (representative traces and pooled data). Approximately 40% of the cells respond to MO (dark cyan), whereas the remaining are insensitive to MO (black). (B and C) Representative traces and (D) pooled data of $[Ca^{2+}]_i$ responses evoked by OXA (10 to 300 μM) and CIS (10 to 300 μM) in CHO cells stably transfected with mouse TRPA1 cDNA. MO (100 μM) is used as positive control. Neither OXA or CIS (both 300 μM) evoke any calcium response in untransfected CHO cells. (D) The effect of OXA and CIS (both 300 μM) is entirely abated by glutathione (GSH; 1 mM). Lines represent average signal of all recorded cells with standard error indicated by dotted lines (A to C). Pooled data represent the comparison of the percentage of cells responding to OXA, OXA plus GSH, CIS, CIS plus GSH, or MO with the total amount of cells analysed. Numbers indicate responding cells/total cells analysed (A and D). *$P < 0.05$ vs. OXA or CIS (both 300 μM); chi-square test.

data obtained in different models of hyperalgesia [14], was evident 60 minutes after dosing (Fig. 3B and C). HC-030031 did not affect mechanical paw-withdrawal threshold in naïve animals (Fig. 3B and C). Finally, pretreatment with capsazepine, at a dose (4 mg/kg, i.p.) that completely reverted mechanical hyperalgesia evoked by the intraplantar injection of capsaicin (20 μg/50 μL/paw)

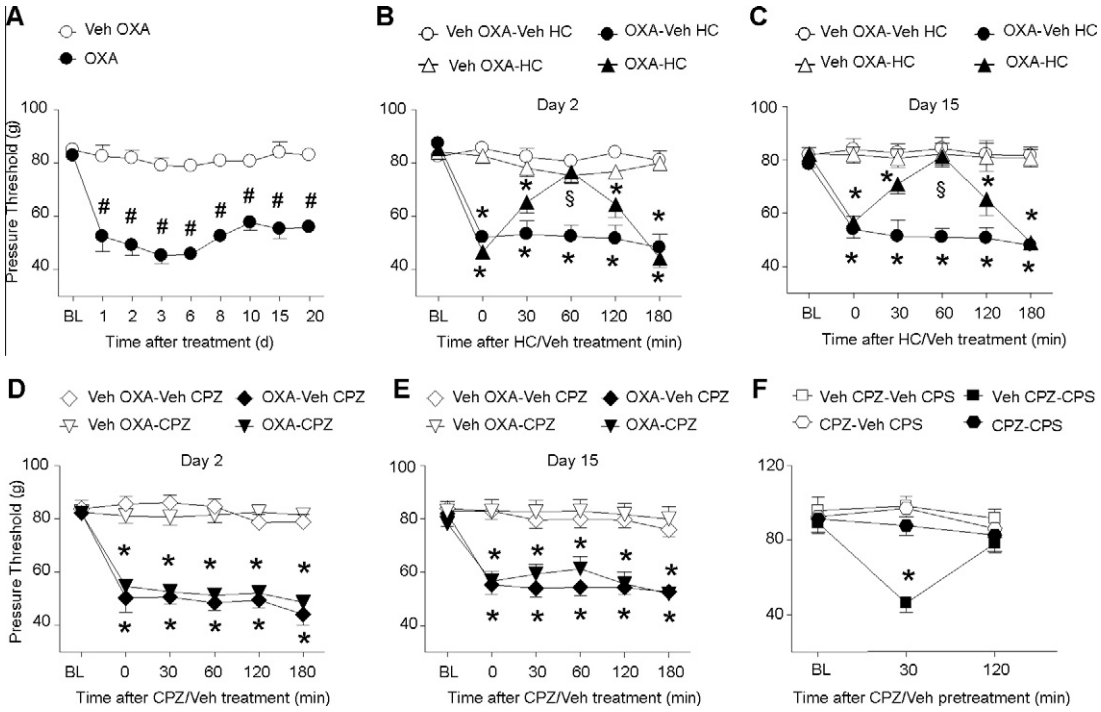

Fig. 3. Oxaliplatin induces mechanical hyperalgesia via TRPA1 activation in rats. A single dose of oxaliplatin (OXA; 2 mg/kg, i.v.) produces a time-dependent reduction in the mechanical paw-withdrawal threshold (A). At days (d) 2 (B) and 15 (C), the treatment with HC-030031 (HC; 100 mg/kg, i.g) completely reverses the mechanical hyperalgesia 60 minutes after dosing. Treatment with capsazepine (CPZ; 4 mg/kg, i.p.), which completely reverses mechanical hyperalgesia induced by capsaicin (CPS; 20 μg/50 μL/paw) (F), does not affect mechanical hyperalgesia induced by OXA either at day 2 (D) or day 15 (E) after treatment. Veh is the vehicle of OXA. Values are mean ± SEM of n = 8–10 rats. $^{\#}P < 0.05$ vs. Veh OXA in A; Student *t* test; $^{*}P < 0.05$ vs. Veh OXA-Veh HC and Veh OXA-HC in B and C or Veh OXA-Veh CPZ and Veh OXA-CPZ in D and E or Veh CPZ-Veh CPS in F; $^{\S}P < 0.05$ vs. OXA-Veh HC in B and C; one-way ANOVA and Bonferroni test. BL, baseline withdrawal threshold.

(Fig. 3F), did not affect mechanical hyperalgesia induced either 2 (Fig. 3D) or 15 (Fig. 3E) days after oxaliplatin administration. Thus, the present data propose a major role for TRPA1 in oxaliplatin-evoked mechanical hyperalgesia in rats, whereas they rule out any contribution by TRPV1.

Following the same protocol of administration [23], we investigated whether oxaliplatin produced thermal (cold) hyperalgesia measured by the immersion of the tail in a cold (10°C) water bath. Indeed, a marked and significant reduction in tail withdrawal latency was observed at days 2 and 3 from oxaliplatin administration (Fig. 4A). This effect of oxaliplatin was reverted by HC-030031, 60 minutes after dosing, with a kinetic similar to the inhibition of mechanical hyperalgesia (Fig. 4B). HC-030031 did not affect cold sensitivity in naïve animals (Fig. 4B). Therefore, present pharmacological evidence supports a major role for TRPA1 in oxaliplatin-evoked cold hyperalgesia in rats.

3.4. Oxaliplatin-evoked mechanical allodynia and cold hypersensitivity in mice are mediated by TRPA1 activation

The administration of a single dose of oxaliplatin (3 mg/kg, i.p.) produced, as reported previously [18], an early-developing reduction in mechanical nociceptive threshold assayed by the Von Frey hair test in C57BL/6 mice. The reduction from baseline value was significant already at day 3 and persisted until day 15 to 20 after drug administration (Fig. 5A). The same experimental paradigm was applied to *Trpa1*$^{+/+}$ or *Trpa1*$^{-/-}$ mice. Oxaliplatin treatment produced mechanical allodynia in *Trpa1*$^{+/+}$ mice (Fig. 5C), with a time course similar to that observed in C57BL/6 mice. The allodynic effect of oxaliplatin was completely absent in *Trpa1*$^{-/-}$ mice (Fig. 5C). It should be noted that the response to oxaliplatin in *Trpa1*$^{-/-}$ mice was superimposable to the responses observed in both *Trpa1*$^{-/-}$ and *Trpa1*$^{+/+}$ mice treated with the oxaliplatin vehicle (Fig. 5C).

As shown elsewhere [17], C57BL/6 mice injected with a single dose of oxaliplatin (3 mg/kg, i.p.) showed early developing cold hypersensitivity, which was assayed by the time spent licking the hind paw after acetone application for cooling stimulation. The increase in cold sensitivity peaked at day 3 and returned to baseline 15 days after oxaliplatin administration (Fig. 5B). A similar response was observed in *Trpa1*$^{+/+}$ mice, whereas no cold hypersensitivity was seen in *Trpa1*$^{-/-}$ mice (Fig. 5D). As shown for mechanical allodynia, responses to cold stimulus after the vehicle of oxaliplatin were similar in *Trpa1*$^{-/-}$ and *Trpa1*$^{+/+}$ mice, and neither were different from those produced by oxaliplatin in *Trpa1*$^{-/-}$ mice (Fig. 5D). Capsazepine, administered at a dosage (30 mg/kg, i.p.) that was previously reported to inhibit TRPM8 [48], did not produce any significant reduction in cold allodynia in mice (Supplementary Fig. 3).

As previously reported [9], 5 weeks after repeated (3 times per week for 5 weeks) administration of cisplatin (2 mg/kg, i.p.) C57BL/6 mice developed mechanical allodynia (Fig. 5E). Following the same experimental protocol, we found that mechanical allodynia, evoked by cisplatin in *Trpa1*$^{+/+}$, was significantly reduced in *Trpa1*$^{-/-}$ mice (Fig. 5F). Genetic findings indicate that TRPA1 is necessary and sufficient to produce mechanical and cold allodynia after oxaliplatin administration in mice, and that TRPA1 also contributes markedly to cisplatin mechanical allodynia.

3.5. Messenger RNA TRPA1 expression

Finally, we evaluated the expression of the messenger RNA of TRPA1 (TRPA1 mRNA) in mouse DRG after 1 single in vivo administration of oxaliplatin (3 mg/kg, i.p.). mRNA TRPA1 was moderately and significantly increased only 6 hours after oxaliplatin treatment, but no increase was observed after 6 hours. Thus, oxaliplatin only transiently and moderately increases mRNA TRPA1 expression in DRG neurons (Fig. 6).

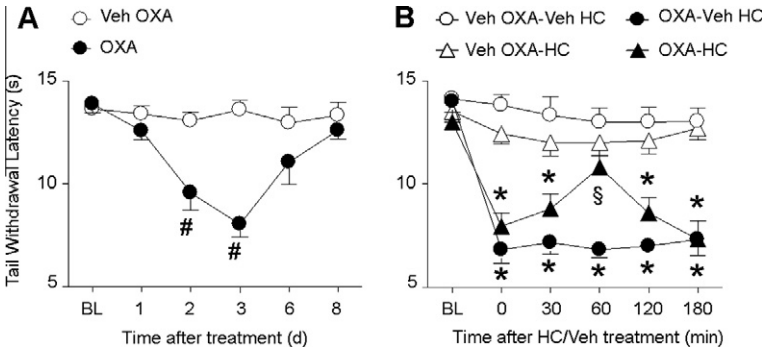

Fig. 4. Oxaliplatin induces cold allodynia via TRPA1 activation in rats. A single dose of oxaliplatin (OXA; 2 mg/kg, i.v.) produces a time-dependent reduction in the tail withdrawal latency to cold (10°C) (A). At day (d) 2, treatment with HC-030031 (HC; 100 mg/kg i.g) completely reverses the cold (B) allodynia for 60 minutes after dosing. Veh is the vehicle of OXA. Values are mean ± SEM of n = 8 to 10 rats. $^{\#}P < 0.05$ vs. Veh OXA in A; Student *t* test; $^{*}P < 0.05$ vs. Veh OXA-Veh HC and Veh OXA-HC in B; $^{\S}P < 0.05$ vs. OXA-Veh HC in B; one-way ANOVA and Bonferroni test. BL, baseline withdrawal threshold.

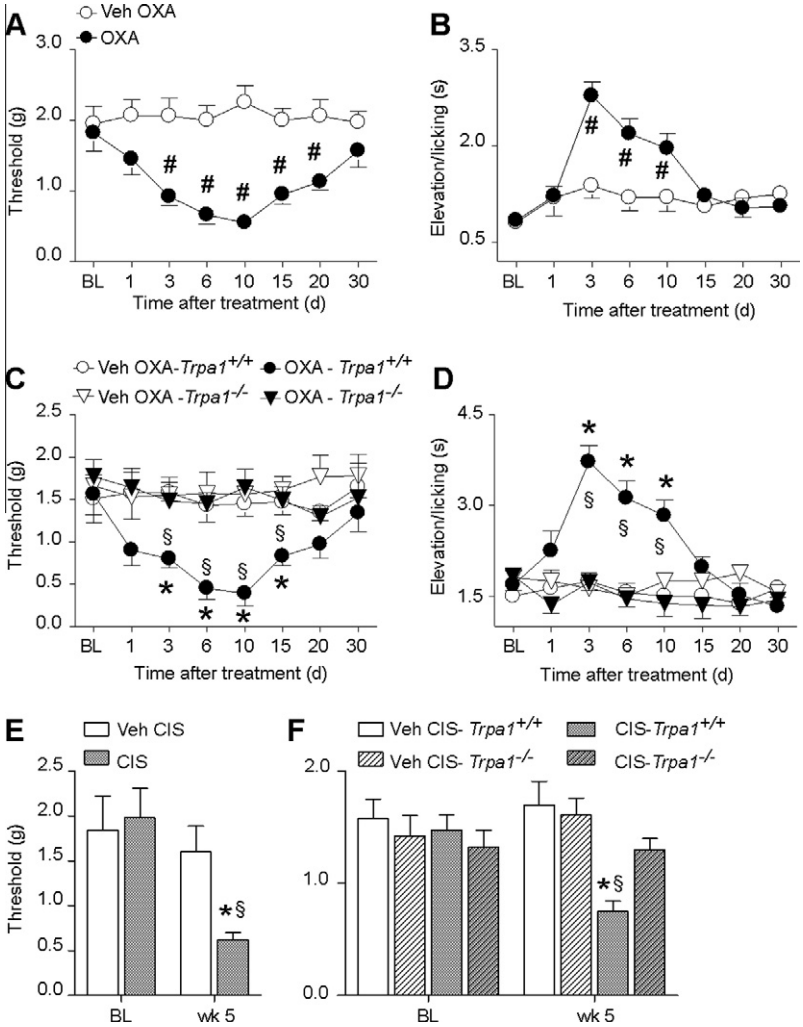

Fig. 5. Oxaliplatin induces mechanical allodynia and cold hypersensitivity via TRPA1 activation in mice. The administration of a single dose of oxaliplatin (OXA; 3 mg/kg, i.p.) induces in C57BL/6 mice a time-dependent reduction in mechanical nociceptive threshold (A), and cold allodynia (B) with a maximum effect at days (d) 10 and 3 after OXA administration, respectively. The development of mechanical (C) and cold (D) allodynia observed in *Trpa1*$^{+/+}$ mice after OXA treatment is completely absent in *Trpa1*$^{-/-}$ mice. (E) Repeated doses of cisplatin (CIS; 2 mg/kg), given 3 times per week throughout 5 weeks, induce a significant reduction in mechanical nociceptive threshold in C57BL/6 mice. (F) The mechanical allodynia evoked by the same treatment in *Trpa1*$^{+/+}$ was significantly reduced in *Trpa1*$^{-/-}$ mice. Veh is the vehicle of OXA or CIS. Values are mean ± SEM of n = 8 to 10 mice. $^{\#}P < 0.05$ vs. Veh OXA and Veh CIS in A and B; Student *t* test; $^{*}P < 0.05$ vs. Veh OXA-*Trpa1*$^{+/+}$ in C and D or vs. CIS (BL) and CIS-*Trpa1*$^{+/+}$ (BL) in E and F and $^{\S}P < 0.05$ vs. OXA-*Trpa1*$^{-/-}$ in C and D or vs. Veh CIS (week 5) and CIS-*Trpa1*$^{-/-}$ (week 5) in E and F; one-way ANOVA and Bonferroni test. BL, baseline withdrawal threshold.

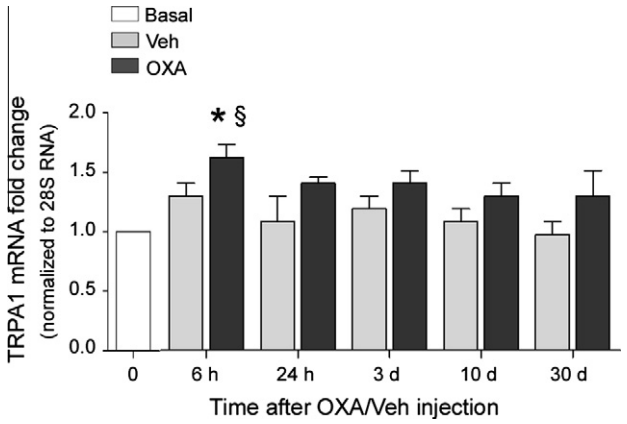

Fig. 6. Effect of oxaliplatin on the expression of TRPA1 mRNA in mouse dorsal root ganglia (DRG). Mice were given an intraperitoneal injection of oxaliplatin (OXA; 3 mg/kg) or its vehicle (Veh; isotonic saline) and DRG were harvested from mice before (baseline, time 0 group) or at different times (h, hours; d, days) after treatment. Total RNA was extracted, and relative TRPA1 mRNA amounts were measured by Taqman real-time polymerase chain reaction (Applied Biosystems, Carlsbad, California, USA). mRNA TRPA1 levels are expressed as fold change over the time 0 group after normalizing to 28S RNA. Each bar is the mean ± SEM of at least 4 mice. $^{*}P < 0.05$ vs. Basal group and $^{\S}P < 0.05$ vs. Veh; one-way ANOVA and Bonferroni test.

4. Discussion

Here, we confirm previous observations obtained in both rats and mice [17,18,23] that a single dose of oxaliplatin produces rapid-onset and time-dependent mechanical and cold hypersensitivity. Pharmacological evidence obtained by using the TRPA1 and TRPV1 channel antagonists (HC-030031 and capsazepine, respectively) suggests that TRPA1 is involved in mechanical (during both the early and the late plateau) and cold hypersensitivity evoked by oxaliplatin in rats. Further and robust support to the hypothesis that TRPA1 plays a major role in mechanical and cold allodynia is derived from genetic data. Indeed, our major finding is represented by the observation that both mechanical and cold hypersensitivity evoked by oxaliplatin administration is completely absent in TRPA1-deficient mice. The contribution of TRPA1 to mechanical hypersensitivity does not seem confined to oxaliplatin, as TRPA1-deficient mice also developed a noticeably reduced mechanical allodynia after cisplatin administration. Thus, TRPA1 appears to be markedly involved in painful neurotoxicity by platinum-based drug.

Various mechanisms have been advocated to explain the bothersome and dose-limiting painful neuropathy associated with the use of oxaliplatin [39]. These include altered intracellular signaling pathways [22], and more recently, altered regulation of the activity of ionic channels, particularly of voltage-gated sodium channels [46,38]. A recent article focused on the role of the TRPV1, TRPM8, ASIC3, and P2X3 receptors on oxaliplatin-induced neuropathy [20]. The hypothesis that TRPM8 contributes to cold hyperalgesia evoked by oxaliplatin administration obtained by using an elevated dose of capsazepine [17], which should inhibit TRPM8 [48], has not been confirmed in our study. In contrast, our findings are in agreement with the observation that IB4-positive somatosensory neurons play a role in oxaliplatin-induced mechanical and cold hypersensitivity [22], given that it has recently been reported that almost half of TRPA1-expressing DRG neurons co-stain with IB4 [25].

Little information has been collected so far on the role of TRPA1 in painful neurotoxicity of platinum-based drugs. A recent investigation has reported a transient increase in TRPA1 mRNA 6 hours after in vitro exposure to oxaliplatin of cultured rat DRG neurons and 6, 24, and 48 hours, but not 12 hours after exposure to cisplatin [43]. More importantly, oxaliplatin or cisplatin administration for 3 weeks to mice in vivo upregulated the TRPA1 mRNA in trigeminal ganglion neurons [43]. However, molecular biology data were not corroborated by functional experiments. The study solely tested the role of TRPV1 that was found to be involved in cisplatin-evoked heat, but not mechanical, hyperalgesia [43]. By using the present experimental protocol, consisting of 1 single injection of oxaliplatin, we also noticed a transient increase in expression of TRPA1 mRNA in mouse DRG homogenates. However, the time course of mechanical and cold hypersensitivity (peak effect was observed at day 10 and day 3, respectively) may hardly be explained by the transient increase in channel mRNA expression, which was seen only 6 hours after oxaliplatin administration. Thus, additional factors must contribute to establishing mechanical and thermal hypersensitivity by oxaliplatin.

Present data have provided evidence to support the contribution of TRPA1 to the neurotoxic effect of platinum-based drugs. Although the mechanism(s) through which TRPA1 contributes to the expression of the hyperalgesic phenotype after exposure to oxaliplatin and cisplatin remain unknown, the following points should be considered. Despite some controversies [6], TRPA1 has been proposed to play a role in cold, mechanical, and chemical nociception [7,12,24,27,28,41]. However, the role of TRPA1 as a sensor of electrophilic and reactive compounds, such as those generated during oxidative stress at sites of tissue injury and inflammation, has been solidly established [2,44]. Indeed, an unprecedented series of oxidative stress byproducts has been reported to gate TRPA1, and through this mechanism produce nociceptive responses and neurogenic inflammation. These reactive agents include H_2O_2 [40], hypochlorite [8], nitroleic acid [44], acrolein [7], 4-hydroxynonenal [45], and other endogenous molecules. There is evidence that oxaliplatin and cisplatin produce oxidative stress [26,32] and, more importantly, that the antioxidants, acetyl-L-carnitine, α-lipoic acid, or vitamin C inhibit oxaliplatin-induced hyperalgesia in rats [22]. Different oxidative stress byproducts, *via* their action on TRPA1, have been reported to recapitulate the ability of oxaliplatin to produce mechanical [5,11,45] and cold [12] hypersensitivity.

In guinea pig pulmonary arteries, which respond to capsaicin through the activation of intramural capsaicin-sensitive sensory nerve terminals and the subsequent release of the vasodilating peptide CGRP [33], both oxaliplatin and cisplatin evoked vasodilatations, which, like that of capsaicin and MO, were mediated by a neurogenic and CGRP-dependent mechanism. Relaxation evoked by oxaliplatin or cisplatin was mediated by TRPA1 because it was selectively inhibited by HC-030031. However, TRPA1-mediated relaxation evoked by oxaliplatin or cisplatin was, in contrast to that of MO or capsaicin, abated by GSH, thus suggesting that the effect of the anticancer drugs on TRPA1 is indirect, and probably mediated by the generation of oxidative stress byproducts. Further evidence that oxaliplatin and cisplatin do not directly stimulate TRPA1 was derived from the observation that both drugs failed to evoke any calcium response in cultured mouse DRG neurons, which constitutively express the channel. However, oxaliplatin and, with lower potency, cisplatin, evoked a GSH-sensitive calcium response in TRPA1-transfected CHO cells, but not in naïve CHO cells. Incubation of GSH with oxaliplatin for 10 minutes (a time period largely exceeding that required to evoke the calcium response in CHO cells or to relax isolated pulmonary arteries) did not affect the concentration of the anticancer drug. This implies that the scavenging action of GSH is not directed primarily to oxaliplatin, but rather to molecules generated after exposure of the tissue/cell to the anticancer drug. Thus, the first requirement for platinum-based drugs for the promotion of calcium response is that the target cell must express the TRPA1 channel. An additional requirement is that either the sensory nerve fiber expressing TRPA1 or the neighboring cells have the capability to generate

oxidative stress byproducts. Present data show indirectly in pulmonary artery tissue and directly in cultured CHO cells that these two preparations have the capability of producing oxidative stress byproducts. In contrast, cultured DRG neurons apparently do not have this capability. In addition, failure of the oxaliplatin metabolite, platinum dichloro diaminocyclohexane [31], to stimulate TRPA1-expressing CHO cells seems to exclude that electrophilic metabolites of the anticancer drug contribute to TRPA1-mediated responses. Experiments performed by co-culturing DRG neurons and naïve CHO cells support the hypothesis that nonneuronal neighboring cells, but not sensory neurons per se, generate the GSH-dependent mechanism responsible for acute activation of neuronal TRPA1.

Thus, we hypothesize that after in vivo exposure to oxaliplatin or cisplatin, ROS and/or reactive aldehydes generated by a variety of nonneuronal cells activate TRPA1 expressed on adjacent sensory nerve terminals, and that this phenomenon contributes to the expression of the hyperalgesic phenotype. Present experiments cannot, however, exclude completely the hypothesis that in vivo DRG neuronal and non-neuronal cells have the ability (lost when they are placed in culture) to produce GSH-sensitive and TRPA1-activating oxidative stress byproducts. In this case, somatosensory neurons themselves, through an autocrine- or paracrine-fashion mechanism, might contribute to TRPA1 activation. Irrespective of the cell type that generates oxidative stress byproducts, this series of events seems to occur early after oxaliplatin administration, but there is indirect evidence that oxidative stress lasts for days after exposure to oxaliplatin. In fact, the antioxidants acetyl L-carnitine, α-lipoic acid, and vitamin C, which profoundly reduced oxaliplatin-evoked mechanical hyperalgesia, were found to be effective even though they were administered on the fifth day after oxaliplatin administration [22]. The mechanism(s) underlying the prolonged increase in TRPA1-mediated hyperalgesia caused by oxaliplatin was not investigated in the present study. However, it has been reported [31] that intracellular or extracellular platinum-bound proteins persist for 50 days after drug administration. We propose that protein modification induced by oxaliplatin is associated with a durable generation of oxidative stress byproducts, which in turn are responsible for TRPA1 activation. Oxaliplatin causes early mechanical hyperalgesia and cold allodynia, whereas cisplatin fails to evoke cold allodynia and produces delayed mechanical hyperalgesia. It is possible that the higher potency or lower doses of oxaliplatin (with respect to cisplatin) required to produce GSH-dependent TRPA1 activation (observed in CHO cells and guinea pig pulmonary artery) contribute to the more precocious occurrence of mechanical hyperalgesia and the generation of cold allodynia by oxaliplatin. However, it is possible that additional and hitherto unknown pharmacokinetic/pharmacodynamic properties of oxaliplatin are responsible for the difference. In conclusion, although present data indicate that TRPA1 contributes to both oxaliplatin- and cisplatin-evoked neuropathic phenotype, the precise mechanisms responsible for the specific, and presumably different, involvement of TRPA1 in mechanical and cold hypersensitivity produced by the two drugs remains to be identified.

A number of potential neuroprotectants have been challenged recently to reduce the severe side effects caused by platinum-based drugs. The most useful prophylactic or curative treatments seem to be blockers of sodium channels, such as amifostine or carbamazepine, and antioxidants such as GSH or α-lipoic acid [36]. However, no evidence-based recommendation can be proposed, given that large confirmatory prospective trials are still lacking [36]. The present findings, which reveal the early and primary role of TRPA1 in oxaliplatin- and cisplatin-evoked mechanical and cold hypersensitivity, suggest that by tackling this novel mechanism, TRPA1 antagonists may protect patients from neurotoxic effects without affecting the anticancer potential of platinum-based drugs and in particular of oxaliplatin.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.pain.2011.02.051.

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