

**Running head: TRPA1 receptor activation mediates MSU inflammation****Title: TRPA1 receptor stimulation by hydrogen peroxide is critical to trigger pain during MSU-induced inflammation**

Gabriela Trevisan<sup>a</sup> (PhD Student), Carin Hoffmeister<sup>b</sup> (PhD Student), Mateus F. Rossato<sup>a</sup> (PhD Student), Sara M. Oliveira<sup>a</sup> (PhD), Mariane A. Silva<sup>a</sup> (PhD Student), Rafael P. Ineu<sup>b</sup> (PhD), Gustavo P. Guerra<sup>c</sup> (PhD), Serena Materazzi<sup>d</sup> (PhD), Camilla Fusi<sup>d</sup> (PhD Student), Romina Nassini<sup>d</sup> (PhD), Pierangelo Geppetti<sup>d</sup> (MD), Juliano Ferreira<sup>a,b,e,\*</sup> (PhD).

<sup>a</sup>Graduate Program in Biological Sciences: Toxicological Biochemistry; <sup>b</sup>Graduate Program in Pharmacology, Federal University of Santa Maria (UFSM), Santa Maria, RS, Brazil.

<sup>c</sup>Federal University of Technology of Paraná, Medianeira Campus, Medianeira, PR, Brazil.

<sup>d</sup>Department of Health Sciences, Clinical Pharmacology Unit, University of Florence, Florence, Italy. <sup>e</sup>Department of Pharmacology, Federal University of Santa Catarina (UFSC), Florianópolis, SC, Brazil.

**\*Corresponding author:** Juliano Ferreira, Department of Pharmacology, Biological Sciences Centre, Block "D"/CCB, Federal University of Santa Catarina, Trindade, Zip code: 88040-900, Florianópolis, SC, Brazil, Phone: +55 48 3721 9491, FAX: +55 48 3337 5479, email: ferreiraj99@gmail.com.

**Financial support:** This study was supported by Conselho Nacional de Desenvolvimento Científico (CNPq) (Brazil) to J.F. and in part by Ente Cassa di Risparmio di Firenze (Italy) to S.M. The authors thank the fellowships from CNPq and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES).

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process which may lead to differences between this version and the Version of Record. Please cite this article as an 'Accepted Article', doi: 10.1002/art.38112

© 2013 American College of Rheumatology  
Received: Jun 13, 2013; Accepted: Jul 25, 2013

**Conflict-of-interest disclosure:** The authors declare no competing financial interests.

P.G. is a member of the editorial boards of *Physiological Reviews*, *Pain* and *Molecular Pain*, and receives research support from Chiesi Farmaceutici, Merck Sharp & Dohme, Italian Institute of Technology, Regione Toscana, Italian Ministry of University and Research, and Ente Cassa di Risparmio di Firenze.

Accepted Article

## Abstract

**Objective:** Gout is a common cause of inflammatory arthritis provoked by the accumulation of monosodium urate (MSU) crystals, but the underlying mechanisms of the pain in acute gout attacks are poorly understood. The aim of the present study was to evaluate the role of transient receptor potential ankyrin 1 (TRPA1) and TRPA1 stimulants, such as hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), in the MSU-induced inflammation model in rodents.

**Methods:** MSU or H<sub>2</sub>O<sub>2</sub> were injected into the hind paw of rodents, or applied in cultured sensory neurons and intracellular calcium *in vitro* and inflammatory or nociceptive responses *in vivo* were evaluated. Pharmacological, genetic or biochemical tools and methods have been used.

**Results:** We found that TRPA1 antagonism, TRPA1 gene deletion or defunctionalization by capsaicin pretreatment of peptidergic TRP-expressing primary sensory neurons markedly decreased MSU-induced nociception and edema. In addition to these neurogenic effects, MSU increased H<sub>2</sub>O<sub>2</sub> levels in the injected tissue, an effect that was abolished by the H<sub>2</sub>O<sub>2</sub>-detoxifying enzyme, catalase. H<sub>2</sub>O<sub>2</sub>, but not MSU, directly stimulated sensory neurons through the activation of TRPA1. Nociceptive responses evoked by MSU or H<sub>2</sub>O<sub>2</sub> injection were attenuated by catalase, the reducing agent dithiothreitol. In addition, MSU injection increased the expression of TRPA1 and TRPV1 as well as enhanced cellular infiltration and IL-1 $\beta$  levels, which were blocked by TRPA1 antagonism.

**Conclusion:** Our results suggest that MSU-injection increases tissue H<sub>2</sub>O<sub>2</sub> thereby stimulating TRPA1 on sensory nerve endings to produce inflammation and nociception. TRPV1, by a hitherto unknown mechanism, also contributes to these responses.

## Introduction

Gout is the most common cause of painful inflammatory arthritis among men and postmenopausal women and, mainly due to an aging population and lifestyle changes, its incidence and prevalence are steadily increasing (1,2). Poorly controlled gout leads to the limitation of activities and significant decrease in health-related quality of life (3). Signs and symptoms of gout are caused by soft tissue deposits of monosodium urate (MSU) crystals, which trigger intense bouts of articular and periarticular inflammation and excruciating pain (1,4). However, the underlying mechanism that from the gout inflammatory process results in sensory symptoms and pain is poorly understood, and accordingly, patients who suffer acute gout attacks are undertreated (1,2).

Some members of the transient receptor potential (TRP) family of ion channels expressed on primary sensory neurons, including the ankyrin 1 (TRPA1) and the vanilloid 1 (TRPV1), have been labeled as thermo-TRP because of their ability to sense changes in temperature (5,6). TRPA1 expressing neurons also contain the neuropeptides, substance P (SP) and neurokinin A (NKA), and the calcitonin gene-related peptide (CGRP), which when released from peripheral terminals cause neurogenic vasodilatation and edema (5,7). We previously demonstrated that TRPV1, TRPV1-positive sensory neurons, and mast cell degranulation contribute to nociceptive and edematogenic responses in experimental animals evoked by MSU in rodents (8).

The observation that high levels of oxidative stress byproducts are found in patients with gout (9), and are produced endogenously after MSU challenge in experimental animals (10-12) suggests a role of oxidative stress in these conditions. In addition to a number of food ingredients (allyl isothiocyanate, found in mustard oil), environmental irritants (acrolein, a volatile and irritant agent present in vehicle exhaust fumes and tear gas), TRPA1 is activated by an unprecedented series of endogenous compounds generated by oxidative stress. These include, hydrogen peroxide ( $H_2O_2$ ), 4-

hydroxynonenal, 4-oxononenal and other compounds (7,13,14), which qualify TRPA1 as a sensor of oxidative stress. There is a large body of evidence indicating that TRPA1 receptor causes inflammatory responses, as well as cold and mechanical hypersensitivity, in models of inflammatory and neuropathic pain (14,15). Thus, the first aim of the present study was to evaluate the contribution of TRPA1 and its activation and sensitization by reactive oxygen species (ROS) in a model of MSU-induced inflammation in rodents.

TRPA1 is usually co-expressed with TRPV1 in a subset of nociceptive neurons, and several studies have described the synergic action of the two channels in different pain conditions (16-18). TRPV1 has already been shown to contribute to hypersensitivity and edema evoked by MSU injection in rodents (8). Thus, the second aim of this study was to explore the cooperation of TRPA1 and TRPV1 in the mechanism of pain-related behavior and inflammation in an MSU-evoked model of inflammation.

## Materials and Methods

### Animals

Adult male Wistar rats (200-300 g) bred in-house, and wild-type (*Trpa1*<sup>+/+</sup>) or TRPA1 deficient mice (*Trpa1*<sup>-/-</sup>) (20-30 g, C57BL/6 background) (19) were used. All protocols employed have been approved by the Ethics Committee of the Federal University of Santa Maria (23081.003640/2009-61) or by the University of Florence (204/2012-B). In addition, the number of animals and intensity of noxious stimuli used were the minimum necessary to demonstrate the consistent effects of the drug treatments in accordance with current ethical guidelines for the investigation of experimental pain in conscious animals (20). Experimenters were blinded to treatment conditions.

### Drugs

If not otherwise indicated, all reagents were from Sigma (Sigma, St Louis, USA) and were dissolved in the appropriate vehicle solutions. HC-030031 was synthesized as previously described (21).

### Preparation and administration of MSU crystals

The synthetic MSU crystals were prepared as previously described (8). The crystals were characterized by polarizing light microscopy and showed clinical morphologic characteristics with a mean length of 12±2 µm, as described previously (8). The preparation was endotoxin free, as determined by the Limulus amoebocyte cell lysate assay (Thermo, Rockford, USA). MSU crystals were suspended in sterile phosphate-buffered saline (PBS) before injection. MSU suspension (0.25 mg/paw) was administered subcutaneously (s.c.) in the plantar surface of the right hind paw of unanesthetized rats (100 µL) or mice (20 µL) as described (8).

### **Evaluation of nociceptive response**

To observe the ongoing nociception, animals were individually placed in transparent glass chambers. After the acclimation period (20 minutes), the amount of time spent flinching or licking the injected paw was timed with a chronometer following s.c. injection, and was used as a measure of ongoing nociception (8,22). Moreover, cold-evoked nociception (cold allodynia) was determined using the acetone evaporative cooling test (23,24), using the following nociceptive scores: (0) no response, (1) quick withdrawal, (2) prolonged withdrawal or repeated flicking of the paw, and (3) repeated flicking and licking of the paw.

### **Determination of inflammatory response**

Edema formation was observed as an increase in paw thickness measured by a digital caliper and calculated as the difference between the basal and the test value (8). Myeloperoxidase (MPO) activity was determined as described before (8). Interleukin-1 beta (IL-1 $\beta$ ) content was assessed using ELISA kit (PeproTech, Rocky Hill, USA). Haematoxylin-eosin (H&E) staining and histological evaluation of inflammatory infiltrated cells (polymorphonuclear leukocytes, PMN) was carried out in a representative area randomly selected by light microscopic analysis with a 20x objective (25).

### **Treatment protocols**

HC-030031 (TRPA1 selective antagonist, 30-300 nmol/paw), camphor (TRPA1 poorly-selective antagonist, 150 nmol/paw), SB-366791 (TRPV1 selective antagonist, 10 nmol/paw), indomethacin (cyclooxygenase inhibitor, 280 nmol/paw) or their vehicle solution (0.1% DMSO in PBS, 100  $\mu$ L/paw,) were s.c. co-injected with MSU (0.25 mg/paw), its vehicle (PBS, 100  $\mu$ L), or the TRPA1 agonists allyl isothiocyanate (AITC, 1 nmol/paw) and H<sub>2</sub>O<sub>2</sub> (3  $\mu$ mol/paw). In another set of experiments, we have orally (p.o.)

administrated the HC-030031 (300  $\mu\text{mol/kg}$ ) or its vehicle (1% DMSO in PBS, 1 mL/kg) 1 hour before the s.c. injection of MSU (0.25 mg/paw) or its vehicle (PBS, 100  $\mu\text{L}$ ). Moreover, we have co-injected the SB-366791 (0.1 nmol/paw) plus HC-030031 (30 nmol/paw) or their vehicle (100  $\mu\text{L/paw}$ , 0.1% DMSO in PBS) with MSU (0.25 mg/paw),  $\text{H}_2\text{O}_2$  (3  $\mu\text{mol/paw}$ ), or its vehicle (PBS, 100  $\mu\text{L}$ ). In a different set of experiments, catalase from bovine liver (300 UI/paw), DTT (20 nmol/paw), or their vehicle (PBS, 100  $\mu\text{L/paw}$ ) were co-injected with MSU (0.25 mg/paw),  $\text{H}_2\text{O}_2$  (3  $\mu\text{mol/paw}$ ), AITC (1 nmol/paw, co-injected only with catalase or vehicle) or its vehicle (PBS, 100  $\mu\text{L}$ ). DTT (20 nmol/paw) was injected 5 minutes before the  $\text{H}_2\text{O}_2$  to prevent any ongoing reaction with  $\text{H}_2\text{O}_2$ . External hind paw temperature was measured before and 10 minutes after the i.pl. injection of  $\text{H}_2\text{O}_2$  (3  $\mu\text{mol/paw}$ ) as described previously (26).

TRPV1 and TRPA1 positive sensory fibers were ablated as described previously (8,27). Briefly, anesthetized animals were desensitized using a perineural injection of capsaicin (2%, 10  $\mu\text{L}$ ) or its vehicle (10% ethanol, 10% Tween 80 in PBS) into the nerve sheath using a microsyringe. Seven days later, MSU (0.25 mg/paw), AITC (positive control, 1 nmol/paw), or their vehicle (PBS, 100  $\mu\text{L/paw}$ ) was s.c. injected. The treatment time and drug doses were chosen on data from previous literature as well as pilot experiments using positive controls (data not shown).

### Western blot analysis

After 7 days of the desensitization protocol or 0.5 or 6 hours of MSU injection (0.5 or 6 hours), rats were euthanized, and the right sciatic nerves or skin hind paw, respectively, were quickly isolated and homogenized in lysis buffer containing protease inhibitors. After centrifugation (3,000  $\times g$ , 30 minutes, 4°C), the supernatant was collected. The protein content was determined using BSA as a standard (28). Then, samples protein (30  $\mu\text{g}$ ) was mixed with loading buffer and boiled for 10 minutes (22,30). The proteins were separated



by electrophoresis on 10% SDS-polyacrylamide gels and transferred to polyvinylidene difluoride membranes. The proteins on the membrane were stained with a solution (0.5% ponceau+1% glacial acetic acid in water), as loading control (22,29,30). After staining, the membranes were dried, scanned, and quantified. Membranes were then processed using the SNAP system (Millipore, USA), blocked with 1% BSA, incubated for 10 minutes with an anti-TRPV1 or anti-TRPA1 antibody (1:150, Santa Cruz Biotechnology, Santa Cruz, USA), washed three times, incubated with an alkaline phosphatase-coupled secondary antibody (1:3000) and visualized with a 5-bromo-4-chloro-3-indolyl phosphate/p-nitro blue tetrazolium system. The membranes were dried, scanned and quantified with the Scion Image PC version of NIH Image. The results were normalized by arbitrarily setting the densitometry of the control group.

#### **Calcium influx in rat dorsal root ganglia (DRG) neurons**

Rat DRG neurons were cultured as previously described (23). Intracellular calcium fluorescence was measured in neurons, as previously reported (21,23). Neurons were exposed to uric acid (100-300  $\mu\text{M}$ ), MSU crystals (0.003-0.100 g/L),  $\text{H}_2\text{O}_2$  (10-5000  $\mu\text{M}$ ), acrolein (30  $\mu\text{M}$ ), capsaicin (0.1  $\mu\text{M}$ ), or their vehicles (buffer solution). HC-030031 and SB-366791 vehicles (used in all the in vitro experiments) were both 1% DMSO. Results are expressed as the increase of  $\text{Ratio}_{340/380}$  over the baseline, normalized to the maximum effect induced by ionomycin (5  $\mu\text{M}$ ) added at the end of the experiment (% Change  $\text{R}_{340/380}$ ).

#### **$\text{H}_2\text{O}_2$ production assay**

To determine the  $\text{H}_2\text{O}_2$  levels in paw skin after MSU s.c. injection, we performed a protocol using the phenol red-HRPO method (31). Briefly, 0.25 to 48 hours after MSU (0.25 mg/paw) or vehicle (PBS, 100  $\mu\text{L}$ ), and 0.25 hours after MSU or vehicle plus HC-

030031 (300 nmol/paw), SB-366791 (10 nmol/paw), or catalase (300 UI/paw) injection, rats were euthanized and hind paw skins were removed. Basal values were assessed in rats not injected. The samples were homogenized in 50 mM phosphate buffer (pH 7.4) containing 5 mM of sodium azide at 4°C for 60 seconds, and the homogenate was centrifuged (12,000  $\times$ g, 20 minutes, 4°C. The supernatant obtained was used to determine the H<sub>2</sub>O<sub>2</sub> levels (31). The H<sub>2</sub>O<sub>2</sub> levels were expressed as  $\mu$ mol of H<sub>2</sub>O<sub>2</sub> on the basis of a standard curve of HRPO-mediated oxidation of phenol red by H<sub>2</sub>O<sub>2</sub>, corrected by protein content (in mg) of the paw skin sample analyzed.

### Statistical analysis

All values are expressed as mean  $\pm$  S.E.M. ED<sub>50</sub> values (i.e., the necessary dose of H<sub>2</sub>O<sub>2</sub> to elicit 50% of the response relative to the control value), which are reported as geometric means accompanied by their respective 95% confidence limits. The percentages of inhibition are reported as the mean  $\pm$  SEM and calculated with the maximum developed responses obtained after injection of MSU, AITC or H<sub>2</sub>O<sub>2</sub> when compared to vehicle-treated animals (control). The statistical significance between groups was assessed by the Student's "t" test (to evaluate statistical significance between 2 groups), in addition to 1- (to assess statistical significance among more than 2 groups) or 2- (to evaluate statistical significance among 2 or more groups in time-course curves) way analysis of variance (ANOVA) when appropriate. Bonferroni's post hoc test was conducted when 1- or 2-way ANOVA was used. *P* values lower than 0.05 (*P* < 0.05) were considered to be significant. The ED<sub>50</sub> values were determined by nonlinear regression analysis with a sigmoid dose-response equation using GraphPad Software version 5.0 (GraphPad Software Inc, La Jolla, CA, USA).

## Results

### TRPA1 activation mediates pain-related behaviors and edema induced by MSU

MSU injection (0.25 mg/paw, s.c.) into the rat paw caused a short-lasting ongoing nociception (from 0 to 15 min), a prolonged cold-evoked allodynia (from 0.25 to 4 hours), and paw edema (from 0.5 to 48 hours) (Figure 1A). According to the time course of the effects produced by MSU administration, time intervals of 0-10 minutes for ongoing nociception, 15 minutes for cold allodynia, and 30 minutes for edema were chosen to investigate their respective mechanisms.

Administration of poorly-selective and selective TRPA1 antagonists, camphor, and HC-030031, respectively, decreased the nociceptive and edematogenic responses evoked by MSU. Local co-administration of HC-030031 (300 nmol/paw) or indomethacin (280 nmol/paw) also markedly inhibited MSU-induced ongoing nociception (84 and 86% inhibition), cold allodynia (100 and 100% inhibition at 0.25 hour), and edema (93 and 87% inhibition at 0.5 hour) at all time points evaluated (Figure 1A and B). The local co-administration of camphor (150 nmol/paw) reduced by 84%, 100%, and 80% of ongoing nociception, cold allodynia, and edema caused by MSU, respectively (Figure 1B). Similar to MSU, s.c. injection of the TRPA1 agonist, AITC, into the rat paw induced ongoing nociception, cold allodynia, and paw edema, all events that were prevented by co-administration with either HC-030031 (300 nmol/paw) or camphor (150 nmol/paw) (Table 1). Neither HC-030031 (300 nmol/paw), camphor (150 nmol/paw, s.c.), nor indomethacin (280 nmol/paw, s.c.) induced nociceptive or edematogenic response *per se* (Table 1). When orally administered, HC-030031 (300  $\mu$ mol/kg, p.o.) was also very effective in preventing MSU-evoked ongoing nociception, cold allodynia and paw edema (93%, 100%, and 75% inhibition, respectively) (Figure 1C). The effects produced by MSU s.c. injection in wild-type mice were markedly reduced in TRPA1-deleted mice (*Trpa1*<sup>-/-</sup>) (ongoing

nociception by 79%; cold allodynia by 100%; edema by 95%), further supporting a major role of TRPA1 channel (Figure 1D).

As the MSU i.pl. was able to increase the TRPA1 and TRPV1 expression 6 h, but not 0.5 h after injection (Figure 2A), we then explored the role of TRPV1- and TRPA1-expressing sensory fibers in pain-related behaviors and edema evoked by MSU. The ability of perineural injection of capsaicin to deplete nociceptive fibers was confirmed by the marked reduction in the density of TRPV1- and TRPA1-positive nerve fibers in the sciatic nerve 7 days after treatment (Figure 2A). Capsaicin pre-treatment practically abolished ongoing nociception, cold allodynia, and edema induced by AITC and MSU (Figure 2B). These results further support the key role of TRPA1 channels, expressed by TRPV1-positive sensory neurons, in MSU-induced nociception and edema.

### **TRPV1 and TRPA1 receptor possess synergic action in MSU-mediated nociception and edema**

As previously observed (8), local injection of SB-366791 (10 nmol/site) significantly reduced MSU-elicited ongoing nociception (98% inhibition) and edema (88% inhibition), but not cold allodynia (Table 1). Furthermore, low doses of HC-030031 (30 nmol/paw) or SB-366791 (0.1 nmol/paw) did not affect MSU-induced nociception or edema when injected alone. However, their combination markedly reduced MSU-induced ongoing nociception (78% inhibition), cold allodynia (82% inhibition), and edema (73% inhibition) in rats (Figure 2C).

### **Inflammatory responses induced by MSU injection were reduced by TRPA1 blockage**

The MPO levels were only increased 6 hours after the MSU s.c. injection, This response was reduced by the co-administration of HC-030031 (300 nmol/paw) or

indomethacin (280 nmol/paw) (Figure 3A). Similar results were observed in the histological analysis. The number of inflammatory infiltrated cells (PMN) was increased 6 hours after MSU challenge and it was reduced by the co-administration of HC-030031 (300 nmol/paw) or indomethacin (280 nmol/paw) (Figure 3B). In addition, the injection of MSU enhanced the levels of IL-1 $\beta$  (6 h after treatment) and this effect was reduced by the co-treatment with HC-030031 (300 nmol/paw) or indomethacin (280 nmol/paw) (Figure 3C).

### **Neither MSU crystals nor uric acid were able to directly activate TRPA1 receptor**

Because of the primary role of TRPA1 in the nociceptive and edematogenic responses by MSU *in vivo*, we asked whether MSU crystals or uric acid could promote calcium influx in rat sensory neurons by TRPA1 activation. Both MSU crystals and uric acid failed to evoke any significant calcium response in 83 of the 134 capsaicin-sensitive DRG neurons which responded to the TRPA1 agonist, acrolein (33 $\pm$ 3% change  $R_{340/380}$ ) (Figure 3D). This finding argues against a direct action of MSU on TRPA1 channel expressed in sensory neurons.

### **MSU induces H<sub>2</sub>O<sub>2</sub> production to stimulate TRPA1 and trigger nociception and edema**

Since MSU stimulates ROS production and TRPA1 is a sensor of oxidative stress (6, 32), we tested whether ROS were involved in TRPA1-mediated responses evoked by MSU. As catalase decomposes H<sub>2</sub>O<sub>2</sub> to H<sub>2</sub>O and O<sub>2</sub> (33), we co-injected the enzyme with MSU. Catalase (300 UI/paw) abolished the development of ongoing nociception (100% inhibition), cold allodynia (100% inhibition) and edema (95% inhibition) (Figure 4A). However, the ongoing nociception, cold allodynia, and edema induced by AITC s.c. paw injection were not reduced by catalase co-administration (data not shown). Next, we found an increase in H<sub>2</sub>O<sub>2</sub> production in the injected tissue 0.25 to 6 hours after MSU

administration (Figure 4B). The  $H_2O_2$  concentration at 0.25 hour was about 7 times greater than baseline values or values measured in vehicle-treated animal tissues (Figure 4B). Moreover, the administration of catalase in a dose that produces antinociceptive and antiedematogenic effects (300 UI/paw), but not HC-030031 (300 nmol/paw) or SB-366791 (10 nmol/paw) prevented the increase in  $H_2O_2$  levels evoked by MSU. Thus, MSU-induced  $H_2O_2$  production seems to be independent of TRPA1 or TRPV1 stimulation (Figure 4C).

As already reported (32,34),  $H_2O_2$  (10-5000  $\mu$ M) produced a concentration-related ( $EC_{50}$  of 566  $\mu$ M and  $E_{max}$  of  $47\pm 4\%$  change  $R_{340/380}$ ) calcium influx in sensory neurons which responded to TRPA1 agonists (Figure 3D).  $H_2O_2$  (500  $\mu$ M) evoked a robust calcium influx (in 59 of 112 capsaicin-sensitive DRG neurons), an effect that was significantly prevented by incubation with HC-030031 (30  $\mu$ M), but not by SB-366791 (3  $\mu$ M) (Figure 3D). HC-030031 and SB-366791 reduced the calcium response, evoked by selective agonists of TRPA1 and TRPV1 receptors, respectively (Figure 3D). Thus, the  $H_2O_2$  generated by MSU may act on sensory neurons mainly activating TRPA1 receptors, thereby causing nociception and edema. In line with this hypothesis, paw injection of  $H_2O_2$  (3  $\mu$ mol/paw, s.c.) produced a transient ongoing nociceptive response and prolonged cold allodynia and edema in rats, with estimated  $ED_{50}$  values of 2.8 (2.1-3.9), 4.7 (2.6-8.7) and 1.2 (0.8-1.8)  $\mu$ mol/paw, respectively (Figure 5A and B). Both HC-030031 (300 nmol/paw) and camphor (150 nmol/paw) markedly inhibited  $H_2O_2$ -evoked ongoing nociception (71% and 75% inhibition), cold allodynia (both 100% inhibition), and edema (96% and 94% inhibition) (Figure 5C). However, the TRPV1 antagonist SB-366791 (10 nmol/site) only decreased ongoing nociception (89% inhibition), without altering cold allodynia or edema induced by  $H_2O_2$  (Figure 5C).

Similar to data obtained with MSU, low doses of HC-030031 (30 nmol/paw) or SB-366791 (0.1 nmol/paw) were unable to alter  $H_2O_2$ -induced nociception or edema when injected alone. In contrast, their combination markedly reduced  $H_2O_2$ -evoked ongoing

Accepted Article  
nociception (81% inhibition), cold allodynia (100% inhibition), and edema (100% inhibition) (Figure 5D). Moreover, H<sub>2</sub>O<sub>2</sub> (s.c. paw injection) increased external paw skin temperature (from 28±1°C before treatment to 32±0.8°C 10 minutes after H<sub>2</sub>O<sub>2</sub> injection, *P*< 0.05, n = 5-6), an effect that could contribute to TRPV1 activation/sensitization (27,35).

● It has been demonstrated that reactive TRPA1 agonists bind to intracellular cysteine residues to activate the channel, an effect prevented by the reducing agent DTT, which reverses cysteine disulfide formation and nitrosylation or oxidization of cysteine sulfhydryls (36,37). Local pre-treatment with DTT (20 nmol/paw) decreased both MSU and H<sub>2</sub>O<sub>2</sub>-induced ongoing nociception (71% and 100% inhibition), cold allodynia (100% and 100% inhibition), and edema (90% and 100% inhibition) (Figure 4D), thus supporting the participation of cysteine residues of TRPA1 receptor in this phenomenon.

## Discussion

Gout is a recurrent cause of acute inflammatory arthritis, which considerably worsens the patient's quality of life (1,2). Despite the vast amount of information about the disease, only limited knowledge on the underlying mechanism of gout pain is available, thus producing an unfavorable impact on current treatments (1,38). In the present study, we obtained biochemical, pharmacological, and genetic data, which suggest a key role of TRPA1 and oxidative stress in pain-like behaviors and edema in a rodent model of MSU-induced inflammation. Recent evidence has underlined the role of TRPA1 in different rodent models of neuropathic and inflammatory pain (7,14,23). Here, we extend the previous findings, showing that both pharmacological inhibition and genetic ablation of the TRPA1 channel abrogate MSU-induced nociception and edema in rodents.

The acute gout flare usually presents as a painful condition associated with the development of cold allodynia and burning pain (3,39), implying the involvement of thermoreceptors found in sensory neurons. Accordingly, we previously identified the contribution of TRPV1 receptor expressed by a subset of primary sensory neurons in ongoing nociception response and edema induced by MSU in rats (8). TRPA1 receptor is co-expressed in about 30% of TRPV1-positive sensory neurons (40). TRPA1-positive neurons also contain neuropeptides, which, upon release from peripheral terminals, mediate neurogenic inflammatory responses. According to these previous findings, we found that ablation of TRPV1-positive sensory fibers by capsaicin treatment markedly reduced the expression of TRPA1-positive nerve fibers and inhibited MSU-induced ongoing nociception, cold allodynia, and edema. Although the initial proposal of TRPA1 as a sensor of cold temperature has been questioned, several recent studies have proposed the contribution of TRPA1 in cold allodynia in a wide range of experimental conditions (15,23,41). In agreement with these conclusions, we found that TRPA1, but not TRPV1, antagonism reduced MSU-induced cold allodynia.



Six hours after injection, MSU induced the local infiltration of PMN leukocytes and the increase of IL-1 $\beta$  levels, two hallmarks of acute gouty attacks (1,42). Moreover, it also enhanced the expression of TRPV1 and TRPA1 receptors. Of note, it has been recently demonstrated that IL-1 is able to increase the expression of TRPA1 cultured synoviocytes (43). Thus, PMN infiltration, IL-1 $\beta$  production, and TRPA1 increased expression induced by MSU seem to be related with edema formation (that was greater at this moment), but not with nociception (that was intense in earlier time points). Furthermore, TRPA1 receptor activation was also important for leukocyte infiltration and cytokine production induced by MSU. Since the blockade of IL-1 $\beta$  has been proposed to be a reliable treatment for acute gouty attacks (42), the reduction of IL-1 $\beta$  by TRPA1 antagonism is a relevant issue.

It has been demonstrated that MSU crystals or uric acid may directly activate different host cell types, in some cases in a manner independent of crystal phagocytosis (10,44,45). The hypothesis that uric acid or MSU crystals directly activate sensory neurons by TRPA1 targeting was excluded by their failure to produce any calcium mobilization in cultured primary sensory neurons. The alternative possibility that uric acid or MSU crystals activate TRPA1 and sensory neurons *via* indirect mechanisms is suggested by the kinetic of the response to MSU. In fact, uric acid or MSU crystals produced a delayed ongoing nociception, which appeared 5 minutes after stimulus administration, while an almost instantaneous response was observed after the injection of AITC or capsaicin (data not shown).

Stimulation of resident or infiltrating proinflammatory cells by MSU crystals and uric acid is known to generate ROS (10-12). We found that MSU injection concomitantly to the appearance of nociception and edema induced a remarkable increase in H<sub>2</sub>O<sub>2</sub> levels within the injected tissue. We have detected that the increase of MSU-induced at the H<sub>2</sub>O<sub>2</sub> concentration peaked at 0.25 hour and it was still significantly different from vehicle up to 6 hours, but in lower levels. Thus, H<sub>2</sub>O<sub>2</sub> levels seem to be pivotal to the early nociception

development, but accessory to the late edema maintenance, which must involve other pro-inflammatory mediators.  $H_2O_2$  has been identified as an endogenous TRPA1 agonist (32,34,46). Thus, it is possible that, following exposure to uric acid or MSU crystals, neighboring cells produce  $H_2O_2$  which, targeting TRPA1 on peptidergic nerve terminals, produces nociceptive and inflammatory responses. While the TRPA1 expressed in neuronal cells seems to be predominant in the MSU-induced responses, non-neuronal cells expressing TRPA1 such as endothelial cells (47) could account, at least in part, for the effects of MSU.

Similarly to direct TRPA1 agonists,  $H_2O_2$  injection provoked an ongoing nociception, cold allodynia, and edema, all phenomena that were observed much earlier than the delayed effects produced by MSU. To further support the role of  $H_2O_2$  we proved the ability of the cell-permeable reducing agent DTT, which, by binding to the cysteine residues, inhibits channel activation (32,36) to protect against the TRPA1-mediated pro-nociceptive and inflammatory responses evoked by MSU. It is worth noting that gout patients have been described as having an increased content of oxidative substances (9).

We previously demonstrated that TRPV1 contributes to nociception and inflammation in a model of acute gout (8). However, present data provide robust evidence that TRPA1 also plays a major role in this process. A combination of low doses of TRPA1 or TRPV1 antagonists which, if administered alone, had no effect, abolished MSU-induced cold allodynia, ongoing nociception, and edema. Previous studies demonstrated that  $H_2O_2$  in mice caused nociception and edema in a manner that is dependent on both TRPA1 and TRPV1 (32,46). In accordance with these findings, in the present study we found that  $H_2O_2$ -elicited nociception and edema were inhibited by a high dose of a TRPA1 antagonist or by the combination of low doses of TRPA1 and TRPV1 antagonists. TRPV1 does not seem directly activated by  $H_2O_2$  (48). However, it is possible that *in vivo* TRPV1 activation/sensitization is produced by mediators/effects evoked by  $H_2O_2$  or TRPA1. This

hypothesis is supported by the finding that H<sub>2</sub>O<sub>2</sub> injection increased paw temperature by about 4°C, a phenomenon which, in turn, could lead to gate TRPV1 (27,35). TRPV1 sensitization by H<sub>2</sub>O<sub>2</sub> (48) and the ensuing enhanced stimulation by heat might also exaggerate TRPA1 activation, as observed in previous (27,35,49) and present studies.

● Thus, it is possible that, it has been shown in other experimental conditions of inflammatory pain (16-18), both TRPV1 and TRPA1 contribute synergistically to the development of inflammatory painful responses evoked by MSU.

In conclusion, H<sub>2</sub>O<sub>2</sub> production by resident cells and the consequent activation of TRPA1 receptor in sensory neurons seem to start the process that generates MSU-induced pain and inflammation. From this initial event, additional mechanisms contributing to the overall inflammatory and sensory response are progressively recruited, in a time-dependent manner. Accordingly, early blockade of TRPA1 in gout might be a reliable pharmacological choice to completely suppress inflammation and pain in acute gout attacks.

## References

1. Terkeltaub R. Update on gout: new therapeutic strategies and options. *Nat Rev Rheumatol* 2010;6:30-8.
2. Richette P, Bardin T. Gout. *Lancet* 2010;375:318-28.
3. Lindsay K, Gow P, Vanderpyl J, Logo P, Dalbeth N. The experience and impact of living with gout: a study of men with chronic gout using a qualitative grounded theory approach. *J Clin Rheumatol* 2011;17:1-6.
4. Busso N, So A. Mechanisms of inflammation in gout. *Arthritis Res Ther* 2010;12:206.
5. Moran MM, McAlexander MA, Biro T, Szallasi A. Transient receptor potential channels as therapeutic targets. *Nat Rev Drug Discov* 2011;10:601-20.
6. Nilius B, Prenen J, Owsianik G. Irritating channels: the case of TRPA1. *J Physiol* 2011;589:1543-9.
7. Baraldi PG, Preti D, Materazzi S, Geppetti P. Transient receptor potential ankyrin 1 (TRPA1) channel as emerging target for novel analgesics and anti-inflammatory agents. *J Med Chem* 2010;53:5085-107.
8. Hoffmeister C, Trevisan G, Rossato MF, de Oliveira SM, Gomez MV, Ferreira J. Role of TRPV1 in nociception and edema induced by monosodium urate crystals in rats. *Pain* 2011;152:1777-88.
9. Krishnan E. Inflammation, oxidative stress and lipids: the risk triad for atherosclerosis in gout. *Rheumatology (Oxford)* 2010;49:1229-38.
10. Falasca GF, Ramachandrala A, Kelley KA, O'Connor CR, Reginato AJ. Superoxide anion production and phagocytosis of crystals by cultured endothelial cells. *Arthritis Rheum* 1993;36:105-16.
11. Abramson S, Hoffstein ST, Weissmann G. Superoxide anion generation by human neutrophils exposed to monosodium urate. *Arthritis Rheum* 1982;25:174-80.

12. Thomas MJ. Urate causes the human polymorphonuclear leukocyte to secrete superoxide. *Free Radic Biol Med* 1992;12:89-91.
13. Trevisani M, Siemens J, Materazzi S, Bautista DM, Nassini R, Campi B, et al. 4-Hydroxynonenal, an endogenous aldehyde, causes pain and neurogenic inflammation through activation of the irritant receptor TRPA1. *Proc Natl Acad Sci U S A* 2007;104:13519-24.
14. Andrade EL, Meotti FC, Calixto JB. TRPA1 antagonists as potential analgesic drugs. *Pharmacol Ther* 2012;133:189-204.
15. del Camino D, Murphy S, Heiry M, Barrett LB, Earley TJ, Cook CA, et al. TRPA1 contributes to cold hypersensitivity. *J Neurosci* 2010;30:15165-74.
16. Schwartz ES, Christianson JA, Chen X, La JH, Davis BM, Albers KM, et al. Synergistic role of TRPV1 and TRPA1 in pancreatic pain and inflammation. *Gastroenterology* 2011;140:1283-1291 e1-2.
17. Salas MM, Hargreaves KM, Akopian AN. TRPA1-mediated responses in trigeminal sensory neurons: interaction between TRPA1 and TRPV1. *Eur J Neurosci* 2009;29:1568-78.
18. Miyamoto T, Dubin AE, Petrus MJ, Patapoutian A. TRPV1 and TRPA1 mediate peripheral nitric oxide-induced nociception in mice. *PLoS One* 2009;4:e7596.
19. Bautista DM, Jordt SE, Nikai T, Tsuruda PR, Read AJ, Poblete J, et al. TRPA1 mediates the inflammatory actions of environmental irritants and proalgesic agents. *Cell* 2006;124:1269-82.
20. Zimmermann M. Ethical guidelines for investigations of experimental pain in conscious animals. *Pain* 1983;16:109-10.
21. Andre E, Campi B, Materazzi S, Trevisani M, Amadesi S, Massi D, et al. Cigarette smoke-induced neurogenic inflammation is mediated by alpha,beta-unsaturated aldehydes and the TRPA1 receptor in rodents. *J Clin Invest* 2008;118:2574-82.

22. Andrade EL, Luiz AP, Ferreira J, Calixto JB. Pronociceptive response elicited by TRPA1 receptor activation in mice. *Neuroscience* 2008;152:511-20.
23. Nassini R, Gees M, Harrison S, De Siena G, Materazzi S, Moretto N, et al. Oxaliplatin elicits mechanical and cold allodynia in rodents via TRPA1 receptor stimulation. *Pain* 2011;152:1621-31.
24. Flatters SJ, Bennett GJ. Ethosuximide reverses paclitaxel- and vincristine-induced painful peripheral neuropathy. *Pain* 2004;109:150-61.
25. Nassini R, Materazzi S, Andre E, Sartiani L, Aldini G, Trevisani M, et al. Acetaminophen, via its reactive metabolite N-acetyl-p-benzo-quinoneimine and transient receptor potential ankyrin-1 stimulation, causes neurogenic inflammation in the airways and other tissues in rodents. *Faseb J* 2010;24:4904-16.
26. Ferreira J, da Silva GL, Calixto JB. Contribution of vanilloid receptors to the overt nociception induced by B2 kinin receptor activation in mice. *Br J Pharmacol* 2004;141:787-94.
27. Moriyama T, Higashi T, Togashi K, Iida T, Segi E, Sugimoto Y, et al. Sensitization of TRPV1 by EP1 and IP reveals peripheral nociceptive mechanism of prostaglandins. *Mol Pain* 2005;1:3.
28. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 1976;72:248-54.
29. Romero-Calvo I, Ocon B, Martinez-Moya P, Suarez MD, Zarzuelo A, Martinez-Augustin O, et al. Reversible Ponceau staining as a loading control alternative to actin in Western blots. *Anal Biochem* 2010;401:318-20.
30. Andre E, Ferreira J, Malheiros A, Yunes RA, Calixto JB. Evidence for the involvement of vanilloid receptor in the antinociception produced by the dialdehydes

unsaturated sesquiterpenes polygodial and drimaniol in rats. *Neuropharmacology* 2004;46:590-7.

31. Nakamura Y, Murakami A, Ohto Y, Torikai K, Tanaka T, Ohigashi H. Suppression of tumor promoter-induced oxidative stress and inflammatory responses in mouse skin by a superoxide generation inhibitor 1'-acetoxychavicol acetate. *Cancer Res* 1998;58:4832-9.

32. Andersson DA, Gentry C, Moss S, Bevan S. Transient receptor potential A1 is a sensory receptor for multiple products of oxidative stress. *J Neurosci* 2008;28:2485-94.

33. Goyal MM, Basak A. Human catalase: looking for complete identity. *Protein Cell* 2011;1:888-97.

34. Sawada Y, Hosokawa H, Matsumura K, Kobayashi S. Activation of transient receptor potential ankyrin 1 by hydrogen peroxide. *Eur J Neurosci* 2008;27:1131-42.

35. Dai Y, Moriyama T, Higashi T, Togashi K, Kobayashi K, Yamanaka H, et al. Proteinase-activated receptor 2-mediated potentiation of transient receptor potential vanilloid subfamily 1 activity reveals a mechanism for proteinase-induced inflammatory pain. *J Neurosci* 2004;24:4293-9.

36. Takahashi N, Mizuno Y, Kozai D, Yamamoto S, Kiyonaka S, Shibata T, et al. Molecular characterization of TRPA1 channel activation by cysteine-reactive inflammatory mediators. *Channels (Austin)* 2008;2:287-98.

37. Macpherson LJ, Dubin AE, Evans MJ, Marr F, Schultz PG, Cravatt BF, et al. Noxious compounds activate TRPA1 ion channels through covalent modification of cysteines. *Nature* 2007;445:541-5.

38. Malawista SE, de Boisfleury AC, Naccache PH. Inflammatory gout: observations over a half-century. *Faseb J* 2011;25:4073-8.

39. Dorwart BB. Thomas sydenham (1624-1689), on gout: 1717. *J Clin Rheumatol* 2004;10:227.

40. Story GM, Peier AM, Reeve AJ, Eid SR, Mosbacher J, Hricik TR, et al. ANKTM1, a TRP-like channel expressed in nociceptive neurons, is activated by cold temperatures. *Cell* 2003;112:819-29.
41. da Costa DS, Meotti FC, Andrade EL, Leal PC, Motta EM, Calixto JB. The involvement of the transient receptor potential A1 (TRPA1) in the maintenance of mechanical and cold hyperalgesia in persistent inflammation. *Pain* 2010;148:431-7.
42. Torres R, Macdonald L, Croll SD, Reinhardt J, Dore A, Stevens S, et al. Hyperalgesia, synovitis and multiple biomarkers of inflammation are suppressed by interleukin 1 inhibition in a novel animal model of gouty arthritis. *Ann Rheum Dis* 2009;68:1602-8.
43. Hatano N, Itoh Y, Suzuki H, Muraki Y, Hayashi H, Onozaki K, et al. Hypoxia-inducible factor-1alpha (HIF1alpha) switches on transient receptor potential ankyrin repeat 1 (TRPA1) gene expression via a hypoxia response element-like motif to modulate cytokine release. *J Biol Chem* 2012;287:31962-72.
44. Ng G, Sharma K, Ward SM, Desrosiers MD, Stephens LA, Schoel WM, et al. Receptor-independent, direct membrane binding leads to cell-surface lipid sorting and Syk kinase activation in dendritic cells. *Immunity* 2008;29:807-18.
45. Rock KL, Kataoka H, Lai JJ. Uric acid as a danger signal in gout and its comorbidities. *Nat Rev Rheumatol* 2012;9:13-23.
46. Keeble JE, Bodkin JV, Liang L, Wodarski R, Davies M, Fernandes ES, et al. Hydrogen peroxide is a novel mediator of inflammatory hyperalgesia, acting via transient receptor potential vanilloid 1-dependent and independent mechanisms. *Pain* 2009;141:135-42.
47. Fernandes ES, Fernandes MA, Keeble JE. The functions of TRPA1 and TRPV1: moving away from sensory nerves. *Br J Pharmacol* 2012;166:510-21.



48. Chuang HH, Lin S. Oxidative challenges sensitize the capsaicin receptor by covalent cysteine modification. *Proc Natl Acad Sci U S A* 2009;106:20097-102.
49. Patil MJ, Jeske NA, Akopian AN. Transient receptor potential V1 regulates activation and modulation of transient receptor potential A1 by Ca<sup>2+</sup>. *Neuroscience* 2010;171:1109-19.

Accepted Article

## Acknowledgements

Master and doctoral fellowships from Conselho Nacional de Desenvolvimento Científico (CNPq), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Programa de Apoio aos Núcleos de Excelência (PRONEX) and Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul (FAPERGS) (Brazil) are acknowledged. Guerra G.P. also acknowledged the PRODOC/CAPES fellowship. We are grateful to Prof. David Julius (UCSF, CA USA) for the kind gift of the TRPA1 deficient mice and Dr. Delia Preti (University of Ferrara, Italy) for providing HC-030031.

## Author Contributions

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published.

Juliano Ferreira had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design: Gabriela Trevisan, Carin Hoffmeister, Mateus Fortes Rossato, Pierangelo Geppetti, Juliano Ferreira.

Acquisition of data: Gabriela Trevisan, Carin Hoffmeister, Mateus Fortes Rossato, Sara Marchesan Oliveira, Mariane Arnoldi da Silva, Romina Nassini, Serena Materazzi, Camila Fusi, Gustavo Petri Guerra, Rafael Porto Ineu.

Analysis and interpretation of data: Gabriela Trevisan, Carin Hoffmeister, Mateus Fortes Rossato, Sara Marchesan Oliveira, Mariane Arnoldi da Silva, Romina Nassini, Serena Materazzi, Camila Fusi, Gustavo Petri Guerra, Rafael Porto Ineu, Pierangelo Geppetti, Juliano Ferreira.

## Figure legends

**Figure 1.** TRPA1 activation mediates monosodium sodium (MSU) crystals-induced ongoing nociception, cold allodynia, and edema in rodents. (A) MSU (0.25 mg/paw)-induced responses were largely reduced by local administration of the TRPA1 receptor selective antagonist HC-030031 (HC, 300 nmol/paw) or indomethacin (INDO, cyclooxygenase inhibitor, 280 nmol/paw). (B) The MSU-induced ongoing nociception, cold allodynia, and edema were reduced by local administration of HC (30 or 300 nmol/paw); and the non-selective TRPA1 antagonist camphor (Cam, 150 nmol/paw). (C) The HC (300  $\mu$ mol/kg, oral route) systemically administered diminished the ongoing nociception, cold allodynia, and edema elicited by MSU (0.25 mg/paw). (D) TRPA1-deficient mice (*Trpa1*<sup>-/-</sup>) presented a diminished MSU-induced ongoing nociceptive response, cold allodynia, and edema. Ongoing nociception, cold allodynia, and edema were measure (in B, C, and D) from 0-10 minutes, 0.25 hour, and 0.5 hour after injection, respectively. Each column represents the mean  $\pm$  S.E.M. of five to seven rats or six to nine mice. The asterisks denote the significance levels. \**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.001 in comparison to vehicle (Veh) treated group (*Trpa1*<sup>+/+</sup>); or #*P* < 0.05, ##*P* < 0.01, or ###*P* < 0.001 difference in comparison to MSU treated group (*Trpa1*<sup>+/+</sup>); 1 or 2-way ANOVA followed by Bonferroni's post hoc test.

**Figure 2.** Monosodium sodium (MSU) crystals injection increased TRPA1 and TRPV1 expression, and ablation of positive TRPA1 and TRPV1 fibers diminished the MSU-elicited responses. Western blot (inset) showing TRPA1 or TRPV1 immunoreactivity in (A) skin hindpaw samples 6 hours after the injection of MSU (0.25 mg/paw, s.c.) or vehicle (Veh) or in (B) right sciatic nerve samples after the injection of capsaicin (CPS, 2%) or Veh. (C) After seven days of intraneural injection of vehicle or CPS; MSU (0.25 mg/paw), AITC (1 nmol/paw), or vehicle were s.c. paw injected. (D) MSU (0.25 mg/paw)-induced responses

were reduced by local administration of HC-030031 (HC, 30 nmol/paw) jointly to SB-366791 (SB, 0.1 nmol/site) in a subdose manner. Ongoing nociception, cold allodynia, and edema were measured from 0-10 minutes, 0.25 hour, and 0.5 hour after injection, respectively. Each column represents the mean  $\pm$  S.E.M. of three to four samples or six to nine rats. \* $P$  < 0.05, \*\*\* $P$  < 0.001 in comparison to Veh treated group (pre-treated with Veh); or # $P$  < 0.05, ### $P$  < 0.001 difference in comparison to MSU or AITC treated group (pre-treated with Veh); Student's "t" test (in A and B), and 1-way ANOVA followed by Bonferroni's post hoc test (in C and D).

**Figure 3.** Monosodium urate (MSU) crystals-induced inflammatory responses were reduced by TRPA1 antagonism. (A) Myeloperoxidase (MPO) level, (B) IL-1 $\beta$  content, or (C) number of inflammatory infiltrated cells per high power fields (HPF, 20x) was increased 6 hours after MSU (0.25 mg/paw) injection, and these effects were reduced by the co-administration of HC-030031 (HC, 300 nmol/paw) or indomethacin (INDO, 280 nmol/paw). (D) Typical traces and pooled data showing that neither MSU (0.1 mg/ml) or Uric Acid (300  $\mu$ M) produced a calcium influx in rat capsaicin-sensitive DRG neurons that normally respond to ACR or H<sub>2</sub>O<sub>2</sub>. HC-030031 (HC, 30  $\mu$ M) significantly reduced the effect evoked by ACR (30  $\mu$ M) or H<sub>2</sub>O<sub>2</sub> (500  $\mu$ M). Veh is the vehicle of the various agonist (buffer), Veh1 is the vehicle of the antagonists (1% DMSO). N.D. (not detectable). Each column represents the mean  $\pm$  S.E.M. of five to seven samples or value of at least 25 neurons. The asterisks denote the significance levels. \*\*\* $P$  < 0.001 in comparison to vehicle treated group (pre-treated with vehicle) or vehicle (Veh or Veh1); or ## $P$  < 0.01, ### $P$  < 0.001 difference in comparison to MSU treated group (pre-treated with vehicle); 1-way ANOVA followed by Bonferroni's post hoc test.

**Figure 4.** MSU induces H<sub>2</sub>O<sub>2</sub> production to stimulate TRPA1 and trigger nociception and edema. (A) MSU (0.25 mg/paw)-induced responses were reduced by the catalase (300 UI/paw) s.c. paw administration. (B) The MSU (0.25 mg/paw) is able to induce the H<sub>2</sub>O<sub>2</sub> production in skin paw at 0.25 to 6 hours after s.c. paw administration. (C) The injection of catalase (300 UI/paw), but not HC-030031 (HC, 300 nmol/paw) or SB-366791 (SB, 10 nmol/paw), was able to reduced the H<sub>2</sub>O<sub>2</sub> content (0.25 hour after injection). (D) MSU (0.25 mg/paw) and H<sub>2</sub>O<sub>2</sub> (3 μmol/paw) elicited ongoing nociception, cold allodynia, and edema were reduced by the cell-permeable reducing agent dithiothreitol (DTT, 20 nmol/paw) s.c. paw injection. Ongoing nociception, cold allodynia, and edema were measure from 0-10 minutes, 0.25 hour, and 0.5 hour after injection, respectively. Each column represents the mean ± S.E.M. of five to seven rats. The asterisks denote the significance levels. \**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.001 in comparison to vehicle (Veh) treated group; or #*P* < 0.05, ###*P* < 0.001 difference in comparison to MSU or H<sub>2</sub>O<sub>2</sub> treated group; 1-way ANOVA followed by Bonferroni's post hoc test.

**Figure 5.** H<sub>2</sub>O<sub>2</sub>-mediated responses were dependent on TRPA1 activation. (A) Time course of H<sub>2</sub>O<sub>2</sub> (3 μmol/paw) elicited ongoing nociception, cold allodynia, and edema in rats. (B) Dose-response curve of ongoing nociception, cold allodynia, and edema induce by H<sub>2</sub>O<sub>2</sub> (0.3, 1, 3, or 10 μmol/paw). (C) TRPA1 antagonists reduced the responses induced by H<sub>2</sub>O<sub>2</sub> s.c. paw injection. The selective antagonist HC-030031 (HC, 300 nmol/paw), or the non-selective TRPA1 antagonist camphor (Cam, 150 nmol/paw), the selective TRPV1 antagonist SB-366791 (SB, 10 nmol/paw), or vehicle (Veh) were s.c. co-injected with H<sub>2</sub>O<sub>2</sub> (3 μmol/paw). (D) The ongoing nociceptive, cold allodynic, and edematogenic responses elicited by H<sub>2</sub>O<sub>2</sub> (3 μmol/paw) were largely reduced by local administration of HC-030031 (HC, 30 nmol/paw) jointly to SB-366791 (SB, 0.1 nmol/site) in a subdose manner. Ongoing nociception, cold allodynia, and edema were measure (in B,

C, and D) from 0-10 minutes, 0.25 hour, and 0.5 hour after injection, respectively. Each column represents the mean  $\pm$  S.E.M. of five to nine rats. The asterisks denote the significance levels. \* $P$  < 0.05, \*\* $P$  < 0.01, \*\*\* $P$  < 0.001 in comparison to vehicle treated group; or # $P$  < 0.05, ### $P$  < 0.001 difference in comparison to H<sub>2</sub>O<sub>2</sub> treated group; 1 or 2-way ANOVA followed by Bonferroni's post hoc test.

Accepted Article

## Tables

Table 1. Controls for the pharmacological treatments.

Treatment	Nociception Time (s)	Cold allodynia (Score)	Δ Paw Thickness (mm)
Vehicle (100 μL/paw)	3±1	0.2±0.2	0.2±0.03
HC-030031 (300 nmol/paw)	4±1	0.2±0.2	0.3±0.05
Camphor (150 nmol/paw)	7±1	0.2±0.2	0.3±0.05
Indomethacin (280 nmol/paw)	5±1	0.2±0.2	0.2±0.05
AITC (1 nmol/paw)	41±5 <sup>***</sup>	2.3±0.3 <sup>*</sup>	1±0.1 <sup>***</sup>
AITC (1 nmol/paw) + HC-030031 (300 nmol/paw)	9±1 <sup>###</sup>	0.2±0.2 <sup>#</sup>	0.5±0.1 <sup>###</sup>
AITC (1 nmol/paw) + Camphor (150 nmol/paw)	13±2 <sup>###</sup>	0.2±0.2 <sup>#</sup>	0.4±0.03 <sup>###</sup>
SB-366791 (10 nmol/paw)	4±1	0.3±0.3	0.2±0.05
MSU (0.25 mg/paw)	37±4 <sup>***</sup>	2.1±0.3 <sup>*</sup>	0.8±0.1 <sup>***</sup>
MSU (0.25 mg/paw) + SB-366791 (10 nmol/paw)	5±4 <sup>###</sup>	1.75±0.5	0.2±0.08 <sup>###</sup>

Data are presented as mean ± SEM. \**P* < 0.05, \*\*\**P* < 0.001, when compared with vehicle, #*P* < 0.05, ###*P* < 0.001 when compared to AITC or MSU treated group; 1-way ANOVA followed by Bonferroni's post hoc test.

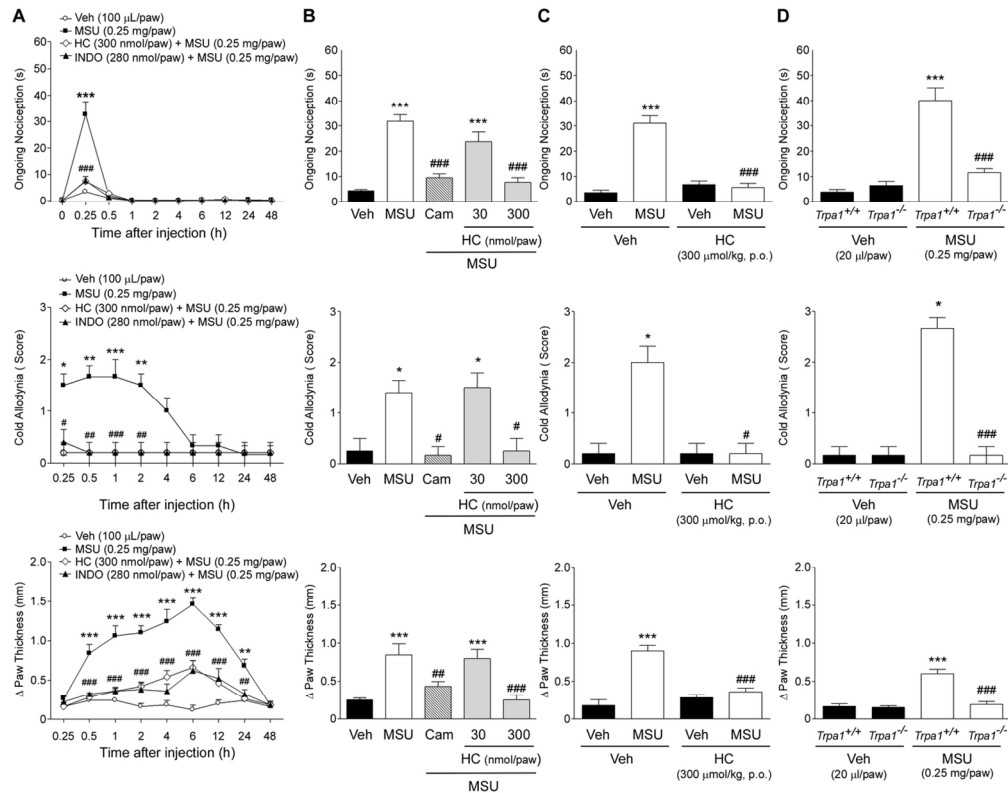


Figure 1. TRPA1 activation mediates monosodium sodium (MSU) crystals-induced ongoing nociception, cold allodynia, and edema in rodents. (A) MSU (0.25 mg/paw)-induced responses were largely reduced by local administration of the TRPA1 receptor selective antagonist HC-030031 (HC, 300 nmol/paw) or indomethacin (INDO, cyclooxygenase inhibitor, 280 nmol/paw). (B) The MSU-induced ongoing nociception, cold allodynia, and edema were reduced by local administration of HC (30 or 300 nmol/paw); and the non-selective TRPA1 antagonist camphor (Cam, 150 nmol/paw). (C) The HC (300 μmol/kg, oral route) systemically administered diminished the ongoing nociception, cold allodynia, and edema elicited by MSU (0.25 mg/paw). (D) TRPA1-deficient mice (*Trpa1*<sup>-/-</sup>) presented a diminished MSU-induced ongoing nociceptive response, cold allodynia, and edema. Ongoing nociception, cold allodynia, and edema were measured (in B, C, and D) from 0-10 minutes, 0.25 hour, and 0.5 hour after injection, respectively. Each column represents the mean ± S.E.M. of five to seven rats or six to nine mice. The asterisks denote the significance levels. \**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.001 in comparison to vehicle (Veh) treated group (*Trpa1*<sup>+/+</sup>); or #*P* < 0.05, ##*P* < 0.01, or ###*P* < 0.001 difference in comparison to MSU treated group (*Trpa1*<sup>+/+</sup>); 1 or 2-way ANOVA followed by Bonferroni's post hoc test. 122x96mm (300 x 300 DPI)



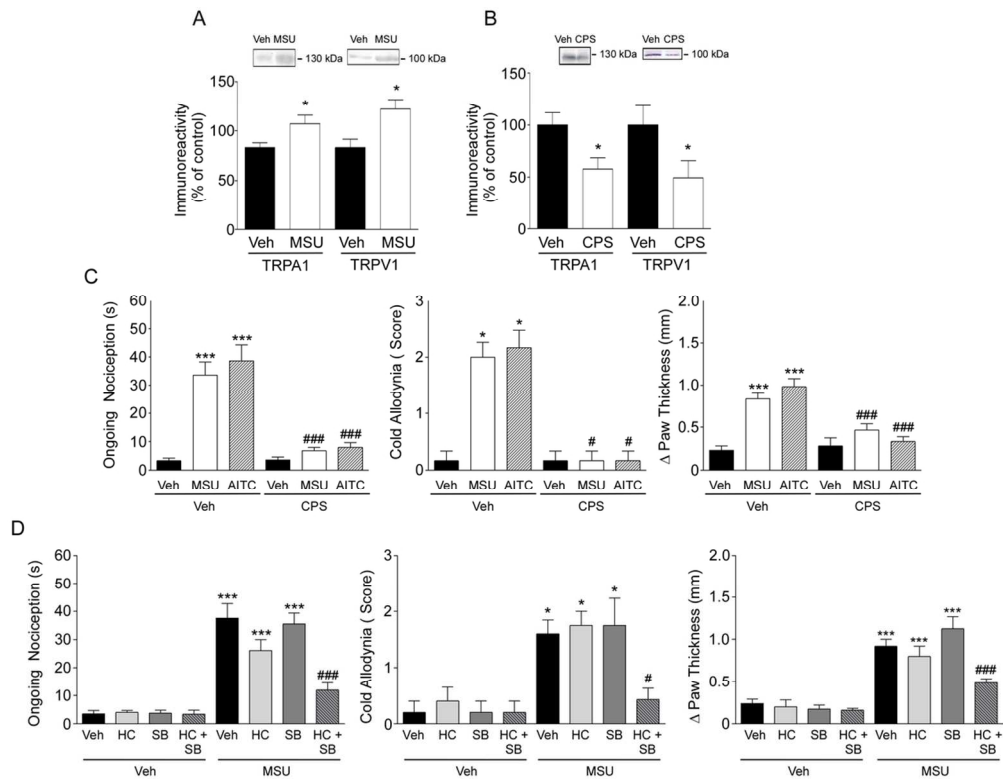


Figure 2. Monosodium sodium (MSU) crystals injection increased TRPA1 and TRPV1 expression, and ablation of positive TRPA1 and TRPV1 fibers diminished the MSU-elicited responses. Western blot (inset) showing TRPA1 or TRPV1 immunoreactivity in (A) skin hindpaw samples 6 hours after the injection of MSU (0.25 mg/paw, s.c.) or vehicle (Veh) or in (B) right sciatic nerve samples after the injection of capsaicin (CPS, 2%) or Veh. (C) After seven days of intraneural injection of vehicle or CPS; MSU (0.25 mg/paw), AITC (1 nmol/paw), or vehicle were s.c. paw injected. (D) MSU (0.25 mg/paw)-induced responses were reduced by local administration of HC-030031 (HC, 30 nmol/paw) jointly to SB-366791 (SB, 0.1 nmol/site) in a subdose manner. Ongoing nociception, cold allodynia, and edema were measured from 0-10 minutes, 0.25 hour, and 0.5 hour after injection, respectively. Each column represents the mean  $\pm$  S.E.M. of three to four samples or six to nine rats. \* $P < 0.05$ , \*\*\* $P < 0.001$  in comparison to Veh treated group (pre-treated with Veh); or # $P < 0.05$ , ### $P < 0.001$  difference in comparison to MSU or AITC treated group (pre-treated with Veh); Student's "t" test (in A and B), and 1-way ANOVA followed by Bonferroni's post hoc test (in C and D).  
107x83mm (300 x 300 DPI)

Acc

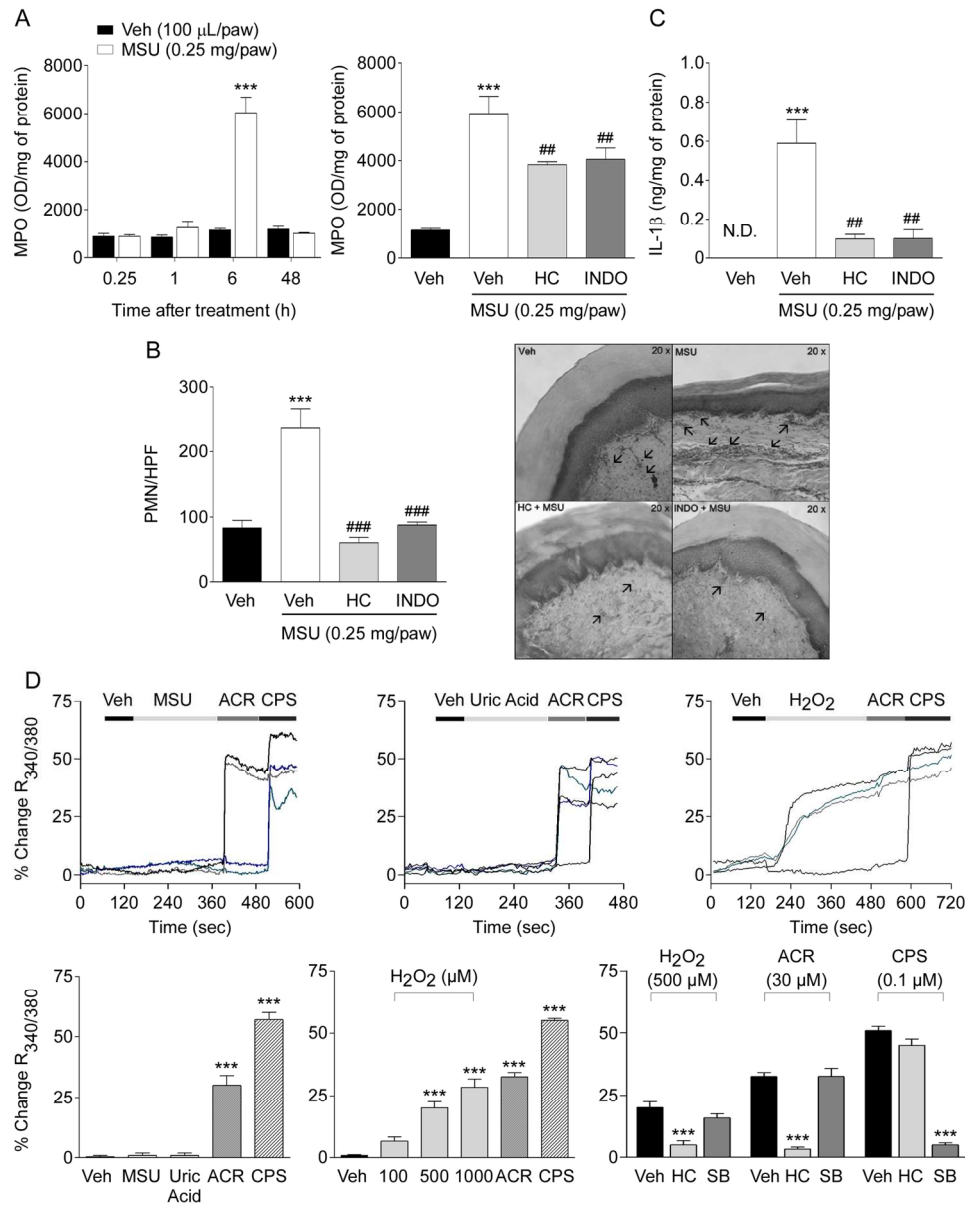


Figure 3. Monosodium urate (MSU) crystals-induced inflammatory responses were reduced by TRPA1 antagonism. (A) Myeloperoxidase (MPO) level, (B) IL-1 $\beta$  content, or (C) number of inflammatory infiltrated cells per high power fields (HPF, 20x) was increased 6 hours after MSU (0.25 mg/paw) injection, and these effects were reduced by the co-administration of HC-030031 (HC, 300 nmol/paw) or indomethacin (INDO, 280 nmol/paw). (D) Typical traces and pooled data showing that neither MSU (0.1 mg/ml) or Uric Acid (300  $\mu$ M) produced a calcium influx in rat capsaicin-sensitive DRG neurons that normally respond to ACR or H<sub>2</sub>O<sub>2</sub>. HC-030031 (HC, 30  $\mu$ M) significantly reduced the effect evoked by ACR (30  $\mu$ M) or H<sub>2</sub>O<sub>2</sub> (500  $\mu$ M). Veh is the vehicle of the various agonist (buffer), Veh1 is the vehicle of the antagonists (1% DMSO). N.D. (not detectable). Each column represents the mean  $\pm$  S.E.M. of five to seven samples or value of at least 25 neurons. The asterisks denote the significance levels. \*\*\* $P$  < 0.001 in comparison to vehicle treated group (pre-treated with vehicle) or vehicle (Veh or Veh1); or ## $P$  < 0.01, ### $P$  < 0.001 difference in comparison to MSU treated group (pre-treated with vehicle); 1-way ANOVA followed by Bonferroni's post hoc test. 144x181mm (300 x 300 DPI)

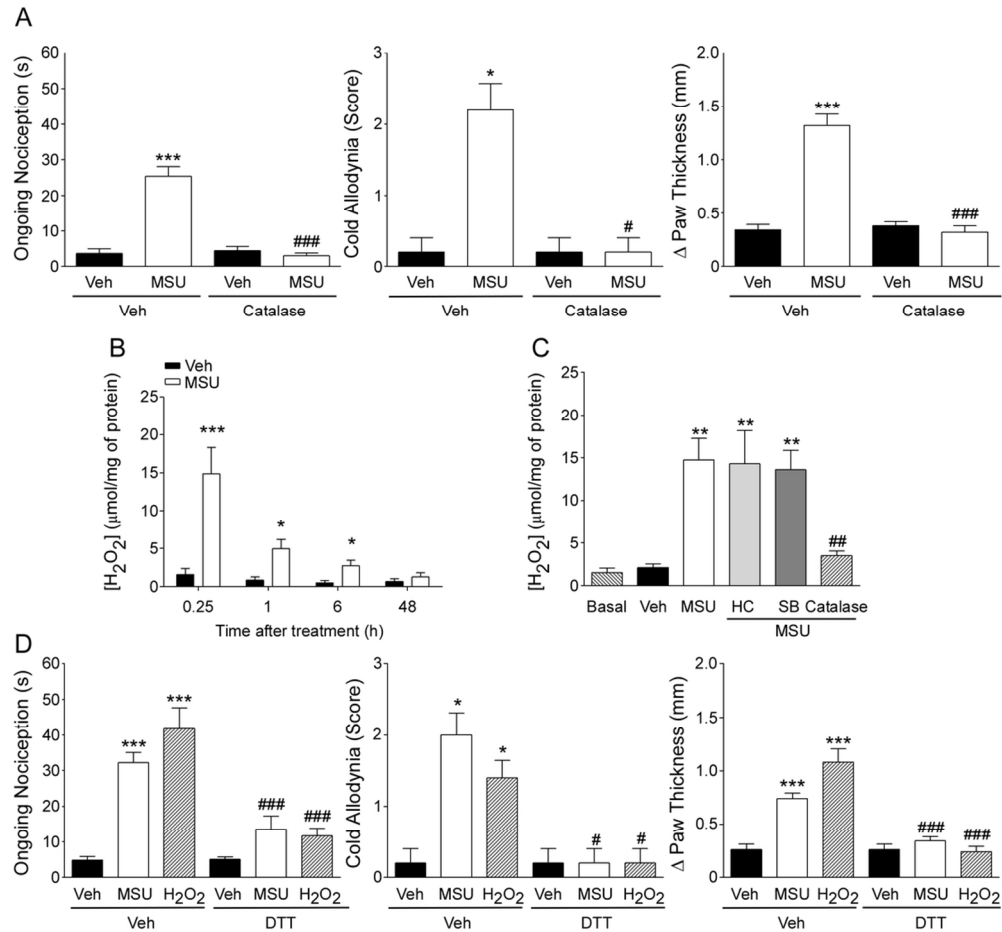


Figure 4. MSU induces H<sub>2</sub>O<sub>2</sub> production to stimulate TRPA1 and trigger nociception and edema. (A) MSU (0.25 mg/paw)-induced responses were reduced by the catalase (300 UI/paw) s.c. paw administration. (B) The MSU (0.25 mg/paw) is able to induce the H<sub>2</sub>O<sub>2</sub> production in skin paw at 0.25 to 6 hours after s.c. paw administration. (C) The injection of catalase (300 UI/paw), but not HC-030031 (HC, 300 nmol/paw) or SB-366791 (SB, 10 nmol/paw), was able to reduced the H<sub>2</sub>O<sub>2</sub> content (0.25 hour after injection). (D) MSU (0.25 mg/paw) and H<sub>2</sub>O<sub>2</sub> (3 μmol/paw) elicited ongoing nociception, cold allodynia, and edema were reduced by the cell-permeable reducing agent dithiothreitol (DTT, 20 nmol/paw) s.c. paw injection. Ongoing nociception, cold allodynia, and edema were measure from 0-10 minutes, 0.25 hour, and 0.5 hour after injection, respectively. Each column represents the mean ± S.E.M. of five to seven rats. The asterisks denote the significance levels. \*P < 0.05, \*\*P < 0.01, \*\*\*P < 0.001 in comparison to vehicle (Veh) treated group; or #P < 0.05, ###P < 0.001 difference in comparison to MSU or H<sub>2</sub>O<sub>2</sub> treated group; 1-way ANOVA followed by Bonferroni's post hoc test.

102x95mm (300 x 300 DPI)

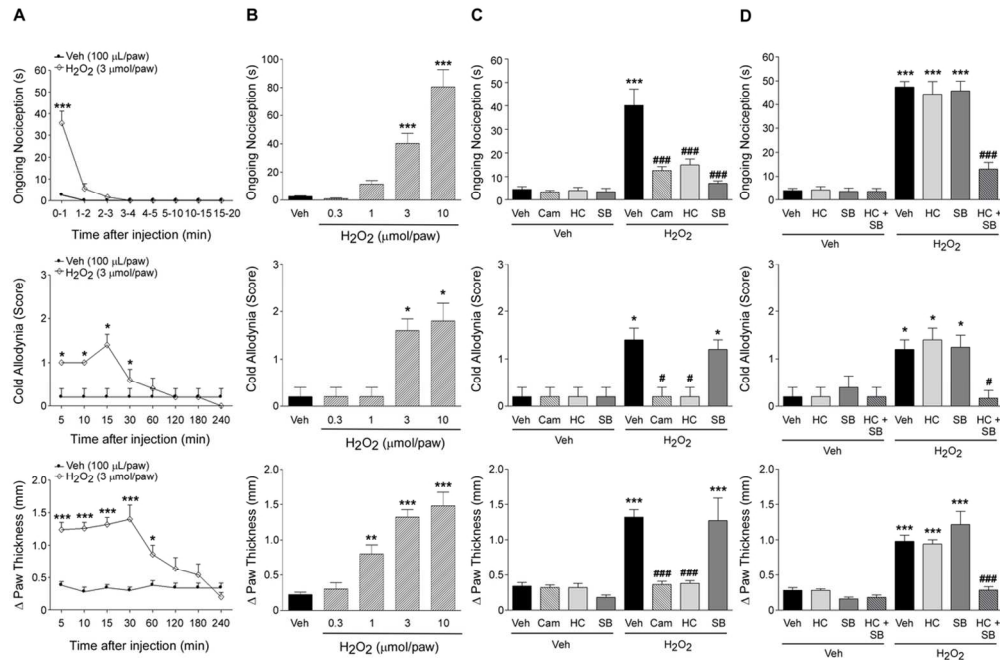


Figure 5. H<sub>2</sub>O<sub>2</sub>-mediated responses were dependent on TRPA1 activation. (A) Time course of H<sub>2</sub>O<sub>2</sub> (3 μmol/paw) elicited ongoing nociception, cold allodynia, and edema in rats. (B) Dose-response curve of ongoing nociception, cold allodynia, and edema induced by H<sub>2</sub>O<sub>2</sub> (0.3, 1, 3, or 10 μmol/paw). (C) TRPA1 antagonists reduced the responses induced by H<sub>2</sub>O<sub>2</sub> s.c. paw injection. The selective antagonist HC-030031 (HC, 300 nmol/paw), or the non-selective TRPA1 antagonist camphor (Cam, 150 nmol/paw), the selective TRPV1 antagonist SB-366791 (SB, 10 nmol/paw), or vehicle (Veh) were s.c. co-injected with H<sub>2</sub>O<sub>2</sub> (3 μmol/paw). (D) The ongoing nociceptive, cold allodynic, and edematogenic responses elicited by H<sub>2</sub>O<sub>2</sub> (3 μmol/paw) were largely reduced by local administration of HC-030031 (HC, 30 nmol/paw) jointly to SB-366791 (SB, 0.1 nmol/site) in a subdose manner. Ongoing nociception, cold allodynia, and edema were measured (in B, C, and D) from 0-10 minutes, 0.25 hour, and 0.5 hour after injection, respectively. Each column represents the mean ± S.E.M. of five to nine rats. The asterisks denote the significance levels. \*P < 0.05, \*\*P < 0.01, \*\*\*P < 0.001 in comparison to vehicle treated group; or #P < 0.05, ###P < 0.001 difference in comparison to H<sub>2</sub>O<sub>2</sub> treated group; 1 or 2-way ANOVA followed by Bonferroni's post hoc test. 109x72mm (300 x 300 DPI)

Acce