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Original Contribution

TRPA1 receptor stimulation by hydrogen peroxide is critical to trigger hyperalgesia and inflammation in a model of acute gout



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ABSTRACT

Acute gout attacks produce severe joint pain and inflammation associated with monosodium urate (MSU) crystals leading to oxidative stress production. The transient potential receptor ankyrin 1 (TRPA1) is expressed by a subpopulation of peptidergic nociceptors and, via its activation by endogenous reactive oxygen species, including hydrogen peroxide (H₂O₂), contributes to pain and neurogenic inflammation. The aim of this study was to investigate the role of TRPA1 in hyperalgesia and inflammation in a model of acute gout attack in rodents. Inflammatory parameters and mechanical hyperalgesia were measured in male Wistar rats and in wild-type (*Trpa1*^{+/+}) or TRPA1-deficient (*Trpa1*^{-/-}) male mice. Animals received intra-articular (ia, ankle) injection of MSU. The role of TRPA1 was assessed by receptor antagonism, gene deletion or expression, sensory fiber defunctionalization, and calcitonin gene-related peptide (CGRP) release. We found that nociceptor defunctionalization, TRPA1 antagonist treatment (via ia or oral administration), and *Trpa1* gene ablation abated hyperalgesia and inflammatory responses (edema, H₂O₂ generation, interleukin-1 β release, and neutrophil infiltration) induced by ia MSU injection. In addition, we showed that MSU evoked generation of H₂O₂ in synovial tissue, which stimulated TRPA1 producing CGRP release and plasma protein extravasation. The MSU-elicited responses were also reduced by the H₂O₂-detoxifying enzyme catalase and the reducing agent dithiothreitol. TRPA1 activation by MSU challenge-generated H₂O₂ mediates the entire inflammatory response in an acute gout attack rodent model, thus strengthening the role of the TRPA1 receptor and H₂O₂ production as potential targets for treatment of acute gout attacks.

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Gout is the principal cause of inflammatory arthritis in men and postmenopausal women. The identification of monosodium urate

Abbreviations: AITC, allyl isothiocyanate; CGRP, calcitonin gene-related peptide; CGRP-LI, CGRP-like immunoreactivity; DTT, dithiothreitol; IL-1 β , interleukin-1 β ; ia, intra-articular; MPO, myeloperoxidase; MSU, monosodium urate; po, oral administration; TRPA1, transient potential receptor ankyrin 1; TRPV1, transient potential receptor vanilloid 1

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(MSU)¹ crystals in the joints of gout patients led to the clinical definition of gout as an inflammatory arthritic disease. However, MSU crystal deposits in joints and periarticular tissues can also exist without associated inflammatory response [1,2]. Acute attacks of gout are accompanied by severe joint pain and articular/periarticular inflammation and, specifically, the presence of MSU crystals in the interior of phagocytic cells [3], which is associated with neutrophil infiltration and production of proinflammatory cytokines, mainly represented by interleukin-1 β (IL-1 β) [1,2].

Regular control using urate-lowering therapies and the reduction of the incidence of acute gout burdens, by nonsteroidal anti-inflammatory drugs or colchicine (standard therapy to control and

prevent acute gout attacks), are the most popular therapies for gout, which, however, may cause significant adverse effects, thus limiting their use. Although new alternatives have been proposed, such as the modified uricases (pegloticase) and interleukin-1 inhibitors [2,4,5], gout patients are still undertreated, and novel strategies for the relief of acute gout attacks with a good efficacy and safety profile are required.

Previous findings that endogenous reactive oxygen species are produced during the process that results in acute gout attack [2,6] and the observation that the antioxidant vitamin C affords some beneficial effects on gout [7–9] have suggested the hypothesis that oxidative stress, by still-unknown mechanisms, contributes to acute gout attacks. The transient receptor potential ankyrin 1 (TRPA1) is a nonselective cation channel activated by endogenous reactive oxygen species, including hydrogen peroxide (H₂O₂) [10]. TRPA1 is coexpressed in sensory neurons along with the hot chili pepper receptor, TRP vanilloid 1 (TRPV1), and the vasodilator and proinflammatory neuropeptide calcitonin gene-related peptide (CGRP) [11–13]. Independent preclinical studies using different rodent models showed that TRPA1 antagonists inhibit nociception and inflammation [11–13].

Previously, we found that H₂O₂ production and subsequent TRPA1 activation contribute significantly to painful and inflammatory responses induced by MSU subcutaneous injection [14]. However, MSU injection into the articular tissue of the ankle seems to represent a more reliable model of acute gout attack than the injection into the subcutaneous tissue, and it is interesting to note that resident cells present in the synovial cavity are considerably different from those in the intraplantar tissue [14–19]. In addition, there are poor data showing the TRPA1 role in articular models of pain and inflammation. Thus, the understanding of TRPA1 participation in the inflammatory and painful processes after ia injection of MSU crystals is critical to guide the use of TRPA1 antagonists in this pathology. The aim of this study was to investigate the role of TRPA1 and H₂O₂ production in the mechanical hyperalgesia and inflammatory responses in an articular model of acute gout attack in rodents

Materials and methods

Ethical statement

All experiments were carried out according to the current guidelines for the care of laboratory animals (European Communities Council (ECC) guidelines for animal care procedures and the Italian legislation (DL 116/92) application of the ECC directive 86/609/EEC) and ethical guidelines for investigations of experimental pain in conscious animals [17]. All protocols were also approved by the Ethics Committees of the Federal University of Santa Maria (Process No. 108/2011(2)) and the University of Florence (Research Permit 143/2008-B and 204/2012-B). To describe the behavioral studies, we have followed the ARRIVE guidelines [18]. Moreover, this study was performed carried out in accordance with the uniform requirements for manuscripts submitted to biomedical journals.

Animals

Experiments were performed using adult male Wistar rats (150–200 g, bred in our vivarium) and littermate wild-type (*Trpa1*^{+/+}) or TRPA1-deficient (*Trpa1*^{-/-}; B6;129P-Trpa1tm1-Kykw/J) mice (20–30 g), which were generated by crossing heterozygous animals on a C57BL/6 background (The Jackson Laboratory, Bar Harbor, ME, USA) [19]. Animals were housed in a controlled-temperature environment in individually ventilated rat or mouse

cages (5 per cage for rats, 10 per cage for mice with wood shaving bedding and no environment enrichment) maintained at 22 ± 1 °C. Animals were maintained on a 12-h light/dark cycle (lights on from 6:00 AM to 6:00 PM) and fed with rodent chow (Puro Lab 22 PB pelleted form, Puro Trato, Rio Grande do Sul, Brazil, for rats or Global Diet 2018, Harlan, Lombardia, Italy, for mice) and tap water ad libitum. Before experiments, animals were allowed to acclimatize to the experimental room for at least 1 h and to their housing environment for at least 72 h after arrival.

Drugs

Unless otherwise indicated, all reagents were from Sigma (St Louis, MO, USA) and were dissolved in the appropriate vehicle solutions. The TRPA1-selective antagonist HC-030031 was synthesized as previously described [20].

Study design

The primary outcome in the behavioral experiments was mechanical hyperalgesia, and the secondary outcome was edema formation after the ia injection of MSU or H₂O₂. These responses were evaluated in the same group of animals for all the treatments. For behavioral experiments, we used a group size of six rats (or six samples) or seven mice for all tests. The group size for each experiment was determined by sample size estimation [21] (analysis of variance sample sizes, desired power 0.8, $\alpha = 0.05$, standard deviation = 4.5 and difference to detect = 6.5 for rats, or standard deviation = 0.25 and difference to detect = 0.33 for mice) for each experiment, based on previous results obtained in our laboratory, in which we have observed mechanical hyperalgesia after MSU ia injection. Each experiment was repeated two or three times. Allocation concealment was not performed before the measure of the baseline threshold of animals, in order to yield groups with similar range basal values in the initial phase of the experiment (before the ia injection of MSU). Experimenters were blinded to the genotype and the drug treatment when performing the tests and to the experimental group when performing analysis. The inclusion and exclusion criteria for the behavioral test were the development of mechanical hyperalgesia and edema formation that were changed by at least 30% compared with the baseline values. No animal or sample was excluded from the analysis. Experiments were conducted between 8:00 AM and 5:00 PM.

Procedures for MSU ia injection and behavioral experiments

Intra-articular injection of MSU crystals

An endotoxin-free MSU crystal suspension in phosphate-buffered saline (PBS) (with a mean length of 12 ± 2 μm) [22], vehicle, or drugs in a volume of 50 or 20 μl (1.25 mg/site) for rats and mice, respectively, was injected into the medial side of the left tibiotarsal joint (ankle) under isoflurane anesthesia [15,16].

Mechanical hyperalgesia

Mechanical hyperalgesia, observed as an increase in nociceptive response, was assessed according to a previously reported procedure and expressed as 50% mechanical paw withdrawal threshold (in g) [23,24].

Edema formation

Edema formation was described as the difference (Δ) between the basal value and the test value measured using a digital caliper [22].

Evaluation of inflammatory cell accumulation and measurement of CGRP and cytokine content

Hematoxylin and eosin (H&E) staining and histological evaluation of emigrated neutrophils was performed after the ia injection of MSU (1.25 mg/site) in articular tissue [25]. Furthermore, various inflammatory parameters were evaluated after MSU injection in synovial lavage samples [26]. The total number of cells was counted using a Neubauer chamber [26]. Myeloperoxidase (MPO) activity was determined as described previously [27]. Protein content in the synovial fluid was determined as described elsewhere [28]. We also measured the CGRP-like immunoreactivity (CGRP-LI) in the synovial fluid as previously described using a commercial ELISA kit (Bertin Pharma, France) [29]. Moreover, the synovial fluid was also assayed for IL-1 β content using an ELISA kit (PeproTech, Rocky Hill, NJ, USA) [14].

Procedures for drug treatment

Here, we observed the antinociceptive and anti-inflammatory effects of drugs using the time points of 1 and 4 h after MSU injection. The 1-h time point was chosen because, at that time, we observed all the nociceptive signs without cellular infiltration; however, at the 4-h time point, we observed the nociceptive and inflammatory signs. The selective and the poorly selective TRPA1 antagonists HC-030031 (300 nmol/site) and camphor (150 nmol/site), respectively, or a vehicle solution (50 μ l/site, 0.1% dimethyl sulfoxide (DMSO) in PBS) was co-injected ia with MSU (1.25 mg/site), the TRPA1 agonist allyl isothiocyanate (AITC; 1 nmol/site), or H₂O₂ (3 μ mol/site), or vehicle. In addition, we tested whether the systemic administration of HC-030031 (300 μ mol/kg, po) or vehicle (1% DMSO in PBS, 1 ml/kg, po) 1 h before the ia injection of MSU (1.25 mg/site) or vehicle (PBS, 50 μ l) reduced MSU-mediated nociception and edema. In addition, MSU crystals (1.25 mg/site, 20 μ l) or PBS (20 μ l/site) was injected ia into *Trpa1*^{-/-} and *Trpa1*^{+/+} mice, and mechanical hyperalgesia and edema were evaluated as described above.

To explore the considerable role of TRPA1-positive fibers in MSU-induced nociception and edema formation, we also employed an ablation protocol using a perineural injection of capsaicin [22]. Animals were used after 7 days to observe the responses to ia injection of MSU crystals (1.25 mg/site), AITC (1 nmol/site; a TRPA1 agonist used as a positive control), or vehicle (50 μ l/site). In a different set of experiments, catalase (a H₂O₂-detoxifying enzyme) from bovine liver (300 UI/site), dithiothreitol (DTT; 20 nmol/site), apocynin (a NADPH-oxidase inhibitor, 1 μ g/site), a CGRP antagonist (CGRP 8-37, 1 nmol/site), or vehicle (50 μ l/site) was co-injected ia with MSU (1.25 mg/site), H₂O₂ (3 μ mol/site), or vehicle (50 μ l/site). The treatment time and drug doses were based on published data as well as on pilot experiments using positive controls (data not shown). Animals were sacrificed with a high dose of intraperitoneal sodium pentobarbital (200 mg/kg).

Western blot analysis

Western blot analysis was carried out as described previously [14,22]. Ponceau staining served as a loading control. A specific anti-TRPA1 primary antibody (anti-TRPA1 polyclonal antibody; Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used. The results were normalized to the control group densitometry values and expressed as the relative amount of TRPA1 immunoreactivity.

Real-time PCR

Spinal cord and fresh blood were taken from sacrificed mice. Lymphocytes and granulocytes were separated by density gradient centrifugation using Ficoll Paque (GE Healthcare Bio-Sciences AB,

Uppsala, Sweden). Total cellular RNA was extracted from spinal cord by using the TRIZOL method (Invitrogen), cDNA was prepared from total RNA using the iScript cDNA synthesis kit (Bio-Rad, Milan, Italy), and the real-time PCR was performed using Sybr green (SsoAdvanced SYBR Green Supermix, Bio-Rad), to check the TRPA1 mRNA levels compared to 18S rRNA.

Determination of hydrogen peroxide levels

H₂O₂ content in synovial tissue was assessed after ia injection of MSU (1.25 mg/site) or vehicle (50 μ l/site) at different time points (1 and 4 h) using the phenol red–HRPO method [14].

Assessment of synovial production of hydrogen peroxide after MSU challenge in vitro

Briefly, rat knee synovial membrane was removed and assayed as described previously [30]. After a stabilization period (2 h), tissues were incubated with MSU (25 mg/ml) or vehicle (assay buffer). Then, after various time points (0.25 to 4 h), aliquots (100 μ l) were removed for H₂O₂ measurement as described above. We also incubated the synovial membranes with colchicine (10 μ M) or vehicle (assay buffer), and after 1 h, the tissues were treated with MSU (25 mg/ml) or vehicle [31].

Evaluation of catalase (CAT) activity

To evaluate CAT activity, we used the method described before [32]. Briefly, 1 or 4 h after vehicle or MSU injection (with or without catalase co-injection, 300 UI/site), we collected samples of articular lavage using PBS (pH 7.4, 10 mM); and also periarticular tissue was collected and homogenized with PBS (pH 7.4, 10 mM). Both samples were centrifuged at 5000 g, 4 °C, for 10 min. The synovial fluid pellet was separated and resuspended in PBS, and synovial fluid and tissue supernatants were also separated for analysis. The reaction was started by mixing 25- μ l samples with 150 μ l of PBS containing 0.02 mM H₂O₂ at 37 °C. The reaction was monitored every 20 s for 2 min (240 nm). The results were expressed as millimoles of H₂O₂ consumed per minute per microliter of sample (synovial fluid supernatant and pellet) or milligram of protein (tissue supernatant).

Statistical analysis

All results were expressed as the mean \pm SEM. Before statistical significance analysis was performed, data were tested for normality using the Kolmogorov–Smirnov test and for homogeneity using the Bartlett test. Hyperalgesia data were log transformed to meet parametrical assumptions. The difference between two groups at one time point was analyzed by Student's *t* test; differences among three or more groups at one time point were analyzed by one-way analysis of variance (ANOVA) followed by Bonferroni's test; differences among three or more groups at different times were analyzed by two-way ANOVA followed by Bonferroni's test. Statistical analysis was performed using GraphPad Software 5.0 (GraphPad Software, San Diego, CA, USA). The percentage inhibition values were reported as the mean \pm SEM obtained in each experiment in relation to the control values. *P* values less than 0.05 were considered significant. To meet the ANOVA assumptions, the mechanical hyperalgesia data were log transformed before statistical analysis.

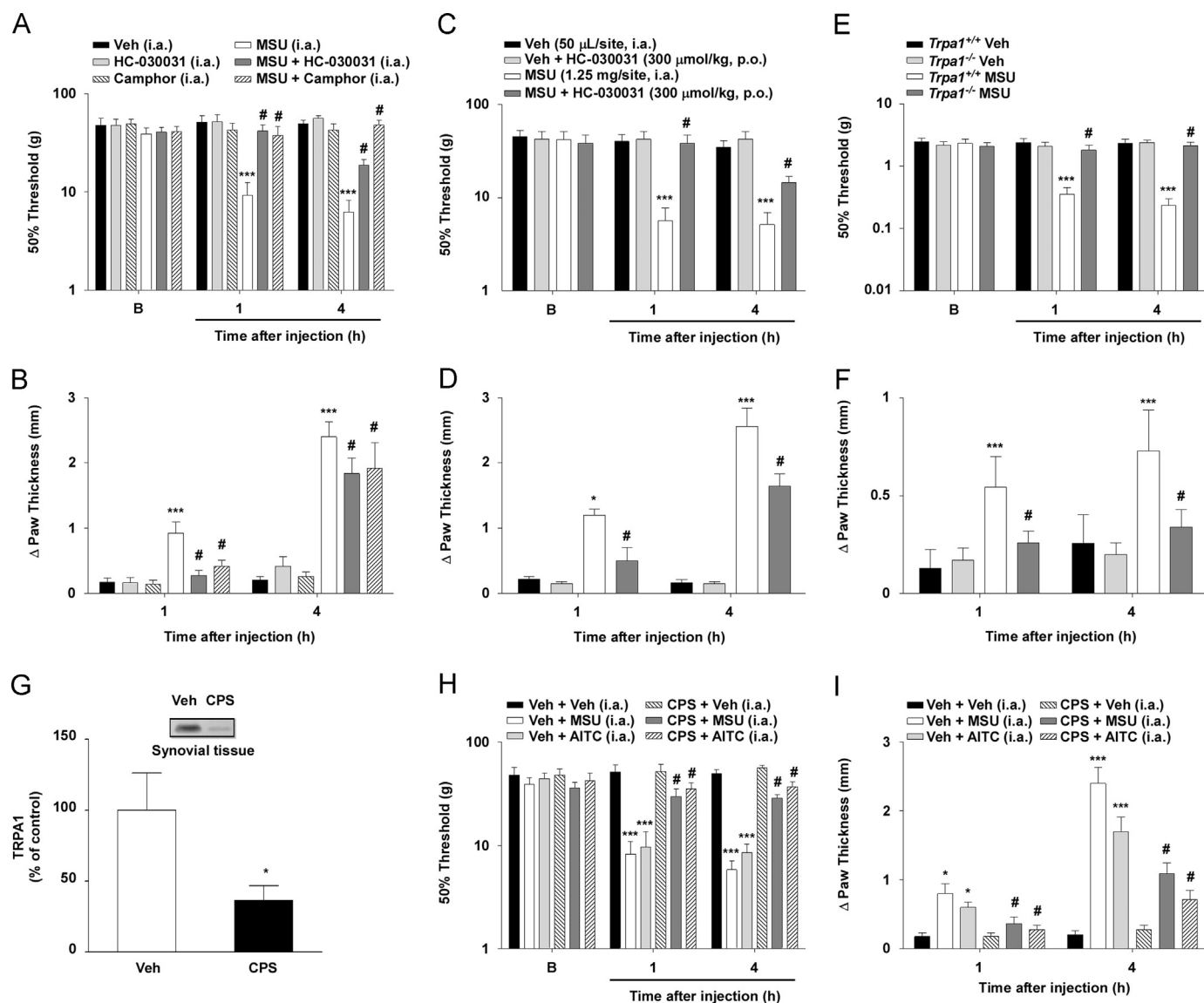


Fig. 1. Inflammatory responses induced by intra-articular (ia) injection of monosodium urate crystals were mediated by TRPA1 channel activation in rodents. (A) Mechanical hyperalgesia and (B) edema caused by ia injection of MSU were reduced by co-injection with the selective and the poorly selective TRPA1 antagonists HC-030031 (300 nmol/site, ia) and camphor (150 nmol/site, ia), respectively. HC-030031 oral pretreatment (300 μ mol/kg, 1 h before MSU injection) also reduced the (C) mechanical hyperalgesia and (D) edema induced by ia MSU injection. TRPA1-deficient mice (*Trpa1*^{-/-}) showed reduced (E) mechanical hyperalgesia and (F) edema formation in response to ia MSU injection compared with *Trpa1*^{+/+} mice. B represents the baseline threshold of animals. Each column represents the mean \pm SEM of six rats or seven mice. Significance levels: **P* < 0.05, ****P* < 0.001 compared with the vehicle (Veh)-treated group (*Trpa1*^{+/+} treated mice in (E) and (F)); #*P* < 0.05 compared with the MSU-treated group (*Trpa1*^{+/+} treated mice in (E) and (F)); two-way ANOVA followed by Bonferroni's post hoc test.

Table 1

Controls for the pharmacological treatments.

Treatment	1 h after treatment		4 h after treatment	
	Mechanical hyperalgesia (PWT in g)	Δ Paw thickness (mm)	Mechanical hyperalgesia (PWT in g)	Δ Paw thickness (mm)
Vehicle (100 μ L/site)	51 \pm 9	0.2 \pm 0.05	50 \pm 5	0.2 \pm 0.06
HC-030031 (300 nmol/site)	52 \pm 9	0.2 \pm 0.05	56 \pm 4	0.3 \pm 0.07
Camphor (150 nmol/site)	43 \pm 7	0.2 \pm 0.07	44 \pm 5	0.3 \pm 0.02
AITC (1 nmol/site)	10 \pm 4***	0.6 \pm 0.07*	9 \pm 2***	1.7 \pm 0.2***
AITC (1 nmol/site) + HC-030031 (300 nmol/site)	51 \pm 5#	0.3 \pm 0.1#	36 \pm 9#	0.9 \pm 0.3#
AITC (1 nmol/site) + camphor (150 nmol/site)	45 \pm 6#	0.3 \pm 0.3#	46 \pm 7#	0.9 \pm 0.2#

Data are presented as the mean \pm SEM. Mechanical hyperalgesia is expressed as 50% mechanical paw withdrawal threshold (PWT in g). Significance was calculated using two-way ANOVA followed by Bonferroni's post hoc test.

* *P* < 0.05, compared with vehicle.

*** *P* < 0.001, compared with vehicle.

P < 0.05, compared to AITC-treated group.

Results

MSU-induced edema and hyperalgesia after ia injection are largely mediated by TRPA1 receptor activation

Before MSU injection, the animals were healthy and without any detectable hyperalgesia or edema (data not shown). Local treatment with the TRPA1-selective antagonist HC-030031 (300 nmol/site, ia) or with the poorly selective TRPA1 antagonist camphor (150 nmol/site, ia) was able to decrease MSU-induced hyperalgesia and also edema 1 and 4 h after treatment (Fig. 1A and B). In addition, ia injection of the selective TRPA1 agonist AITC caused nociceptive and edematogenic responses, which were markedly reduced by the TRPA1 antagonists (HC-030031 or camphor, ia) (Table 1). Oral administration of HC-030031 (300 μ mol/kg, po) also largely reduced the development of MSU-elicited hyperalgesia and edema formation from 1 to 4 h after treatment (Fig. 1C and D). HC or camphor per se did not produce any measurable inflammatory response compared to vehicle (Fig. 1).

Trpa1^{+/+} mice showed mechanical hyperalgesia and edema formation after ia injection of MSU at all evaluated time points. However, *Trpa1*^{-/-} mice presented a marked reduction in MSU-triggered responses (Fig. 1E and F). Further, in naïve rats, ablation of TRPA1-positive nerves by perineural injection of capsaicin reduced TRPA1 channel immunoreactivity in the synovial tissue (64% reduction) 7 days after treatment (Fig. 2A). In addition, ablation of TRPA1-positive fibers was associated with diminished MSU- and AITC-induced hyperalgesia and edema from 1 to 4 h (Fig. 2B and C).

MSU ia injection increases TRPA1 expression in synovial tissue and CGRP release

MSU increased TRPA1 expression 2.5-fold in synovial tissue 4 h, but not 1 h, after ia injection of MSU (Fig. 3A). However, we did not observe any TRPA1 mRNA expression in lymphocytes and granulocytes taken from rodent blood, compared to samples obtained from mouse spinal cord (Fig. 3B).

TRPA1 neuronal activation was assessed by measuring CGRP-LI release by MSU, which produced a 1.5-fold increase in synovial lavage fluid compared to vehicle, and HC-030031 administration reduced CGRP-LI release (Fig. 3C). The H₂O₂-detoxifying enzyme catalase abolished CGRP-LI release after MSU injection, suggesting a role for endogenous H₂O₂ in the TRPA1-mediated MSU response (Fig. 3C).

In addition, the co-injection of the CGRP antagonist (CGRP 8-37) was able to decrease the nociception and edema formation induced by MSU ia injection (Fig. 3D and E).

MSU challenge increased H₂O₂ production and reduced CAT activity

MSU injection (25 mg/ml) at 1 and 4 h increased synovial tissue H₂O₂ levels (Fig. 3F). The MSU challenge also increased synovial membrane H₂O₂ production at various time points (0.25 to 4 h). In addition, pretreatment of the synovial membranes with colchicine (10 μ M for 1 h) reduced the H₂O₂ production (Fig. 3G).

Moreover, MSU ia injection increased the CAT activity in the synovial fluid (pellet and supernatant, after 4 h) and also in the synovial tissue (after 1 and 4 h; Fig. 4A–C). The ia co-injection of exogenous catalase reduced this effect in all measured samples and also increased the CAT activity after 1 h in the synovial fluid samples, compared to vehicle-treated animals (Fig. 4A–C).

The inflammatory responses elicited by ia injection of MSU were possibly mediated by hydrogen peroxide production and subsequent TRPA1 activation

H₂O₂ (3 μ mol/site, ia) injection induced mechanical hyperalgesia and edema. TRPA1 antagonists (HC-030031 and camphor)

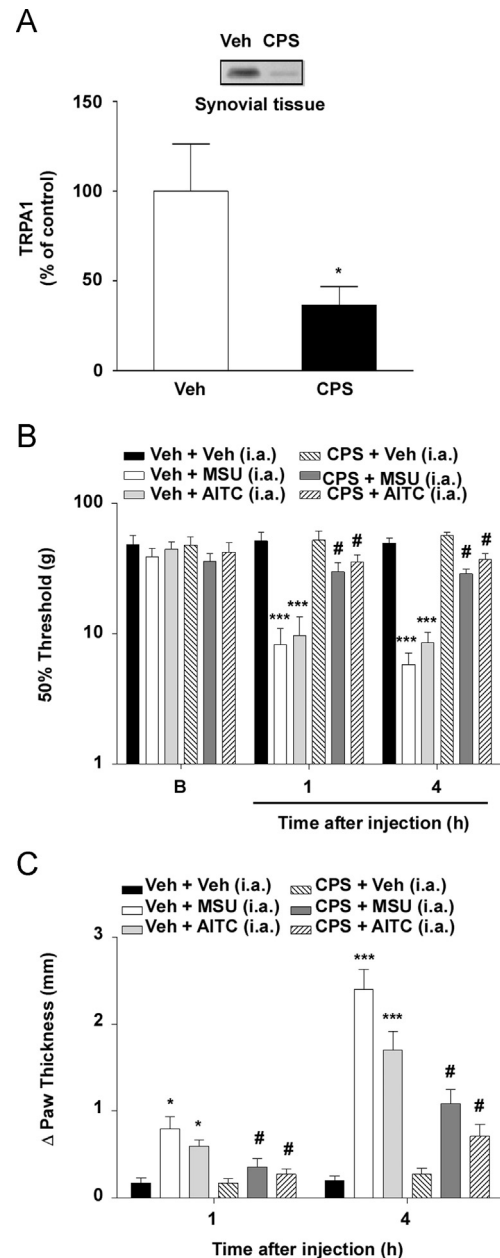


Fig. 2. Ablation of TRPA1-positive fibers largely reduced inflammatory responses elicited by injection of monosodium urate crystals. (A) Western blot (inset) showing TRPA1 immunoreactivity in synovial tissue samples 7 days after the injection of capsaicin (CPS; 2%) or vehicle (Veh). Western blot results are expressed as % of control. The perineural injection of CPS (2%) 7 days before the ia injection of MSU (1.25 mg/site) crystals, the TRPA1 agonist AITC (1 nmol/site), or vehicle reduced the (B) mechanical hyperalgesia and (C) edema formation induced by MSU or AITC ia injection in rats. B represents the baseline threshold of animals. Each column represents the mean \pm SEM of six rats, except for Western blot, for which three to four samples were used. Significance levels: * $P < 0.05$, *** $P < 0.001$ compared with the Veh-treated group (pretreated with Veh); # $P < 0.05$ compared with the MSU- or AITC-treated group (pretreated with Veh); Student's *t* test (in A) or two-way ANOVA followed by Bonferroni's post hoc test (in B and C).

reduced the mechanical hyperalgesia and edema formation induced by ia injection of H₂O₂ after 1 and 4 h (Fig. 4A and B). Catalase (300 UI/site) diminished either MSU- or H₂O₂-induced hyperalgesic and edematogenic responses at 1 and 4 h (Fig. 4C and D). The reducing agent DTT (20 nmol/site), which reverses TRPA1 activation by H₂O₂ in vitro [33], also decreased MSU- or H₂O₂-induced hyperalgesia and edema (Fig. 4E and F). Moreover, the

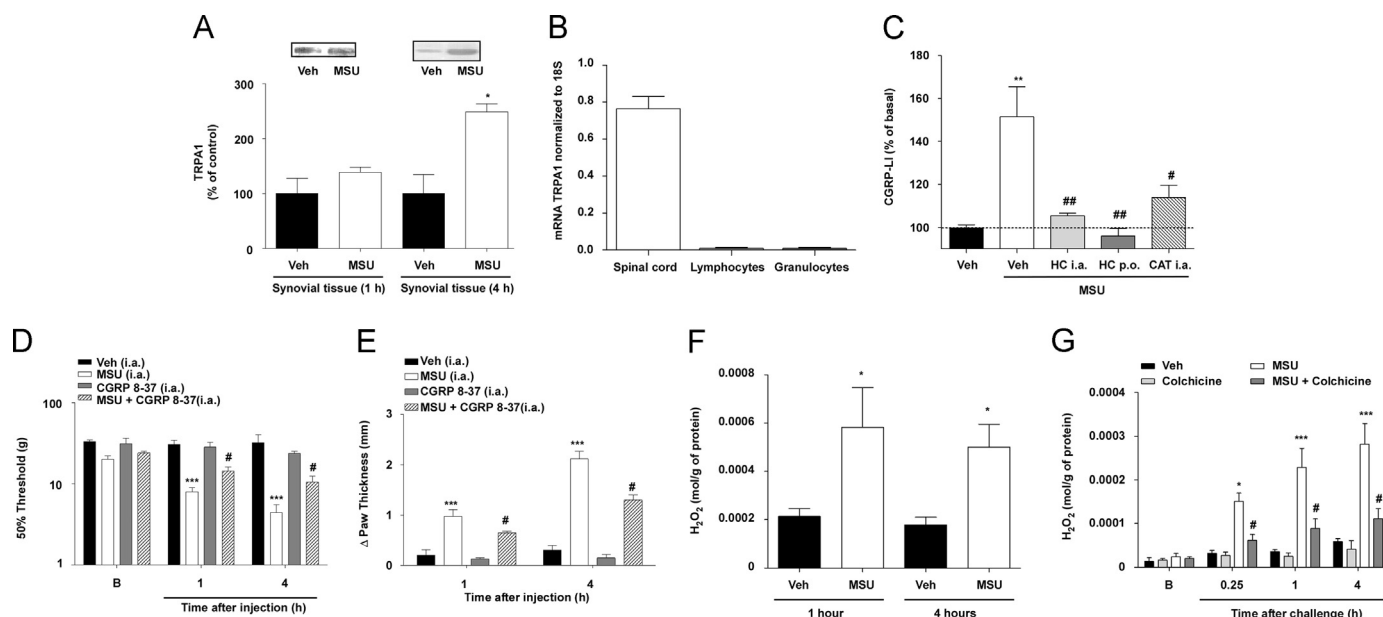


Fig. 3. Monosodium urate crystal-elicited nociceptive and edematogenic responses were accompanied by an increase in TRPA1 expression, release of CGRP, and H_2O_2 production and a reduction in catalase activity by the synovial tissue. (A) Western blot (inset) showing TRPA1 immunoreactivity in synovial tissue at 1 and 4 h after the ia injection of MSU (1.25 mg/site) or vehicle (Veh). Western blot results are expressed % of control. (B) TRPA1 mRNA levels normalized to 18S in spinal cord, lymphocytes, and granulocytes. The expression in lymphocytes and granulocytes is almost zero compared to the TRPA1 expression in mouse spinal cord. (C) Calcitonin gene-related peptide-like immunoreactivity (CGRP-LI) was increased 15 min after MSU (1.25 mg/site) ia injection, an effect reduced by co-injection with HC (300 nmol/site) or CAT (300 UI/site) or by oral pretreatment with HC (300 μ mol/kg, 1 h). The CGRP-LI assay was performed in the synovial lavage samples obtained from pretreated rats. The CGRP antagonist (CGRP 8-37, 1 nmol/site, ia), when co-injected with MSU (1.25 mg/site, ia), decreased the (D) mechanical hyperalgesia and (F) edema formation induced by MSU. (E) The ia injection of MSU (1.25 mg/site) crystals increased H_2O_2 content in synovial tissue 1 or 4 h after administration compared with samples from vehicle (50 μ l/site)-injected animals. (G) Incubation with MSU (25 mg/ml) of rat synovial membranes in vitro enhances the production of H_2O_2 at various time points (0.25 to 4 h), and pretreatment of the membranes with colchicine (10 μ M for 1 h) reduced the H_2O_2 production by rat synovial membranes challenged with MSU (25 mg/ml). B represents the basal level of H_2O_2 production. The data are expressed as the mean \pm SEM of six rats or six samples, except for Western blot, for which three to four samples were used. Significance levels: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with the Veh-treated group; or # $P < 0.05$, ## $P < 0.05$ compared with the MSU-treated group; Student's *t* test (in A and B) and one-way ANOVA (in C and D) or two-way ANOVA followed by Bonferroni's post hoc test (in E, F, and G).

NADPH-oxidase inhibitor apocynin reduced the hyperalgesia and edema caused by MSU ia injection (Fig. 4G and H).

MSU-induced increases in neutrophil infiltration, plasma extravasation, and IL-1 β levels were largely reduced by TRPA1 antagonism or by catalase

We found that MSU (1.25 mg/site, ia) significantly increased the number of emigrated neutrophils in H&E-stained slices of articular tissue at 4 h, but not 1 h, after injection (Fig. 5A and B). The inflammatory effect induced by MSU was reduced by co-injection (300 nmol/site, ia) or oral pretreatment with HC-030031 (300 μ mol/kg, 1 h before; Fig. 5A and B). Finally, HC-030031 or catalase injection reduced the number of neutrophils (Fig. 5C), the MPO activity (Fig. 5D), the plasma extravasation (Fig. 5E), and the increase in IL-1 β content (Fig. 5F) observed in the synovial tissue 4 h after ia MSU challenge. Moreover, CGRP antagonist (CGRP 8-37) and NADPH-oxidase inhibitor (apocynin) injection reduced the number of neutrophils induced by MSU ia injection (Fig. 5C).

Discussion

Hyperalgesia and edema are major symptoms in patients affected by acute gout attacks, and the reduction of pain hypersensitivity and inflammation is a main therapeutic goal for gout treatment [34]. The major finding of this study is that pharmacological blockade or genetic ablation of the TRPA1 channel markedly decreases the mechanical hyperalgesia and the entire inflammatory repertoire produced by ia injection of MSU, a predictive rodent model of acute gout attack. Moreover, we

showed that resident cells from the articular space are important to drive the initial inflammatory process, an effect that we did not observe in our previous study of MSU injection in the intraplantar tissue (subcutaneous). Thus, our data add significant novelty to the understanding of TRPA1 participation in a model of acute gout in rats and mice.

A series of studies reported the contribution of TRPA1 to hyperalgesia and edema in inflammatory pain models [11–13]. TRPA1 is co-expressed with TRPV1 in peptidergic sensory neurons [35]. This implies that the known ability of capsaicin to produce desensitization of TRPV1-expressing neurons results in profound desensitization of TRPA1-positive, peptidergic afferents [36]. In addition, the use of capsaicin as a counterirritant to treat gout pain is probably based on the desensitization properties of the drug [35]. Here, we showed that capsaicin desensitization produced antinociceptive and anti-inflammatory effects by deactivating TRPA1-expressing neurons. The observation that capsaicin treatment decreased TRPA1 immunoreactivity in the synovial tissue may be explained by the ablation of sensory nerve terminals by the desensitizing action caused by intense TRPV1 stimulation.

Furthermore, we observed that the TRPA1 neuronal activation by MSU injection increased CGRP release, and this effect was blocked by TRPA1 antagonism and the use of the H_2O_2 -detoxifying enzyme catalase. In accordance with these data the CGRP antagonist (CGRP 8-37) reduced MSU-induced hyperalgesia and edema, showing that TRPA1 activation produced CGRP release driving inflammation and pain. However, MSU crystals or uric acid do not directly stimulate TRPA1, whereas H_2O_2 promotes TRPA1 activation [10,14]. Injection of MSU (ia) increased the H_2O_2 levels in synovial membranes in vitro, an effect that was reduced by colchicine, indicating a role of MSU phagocytosis in this process.

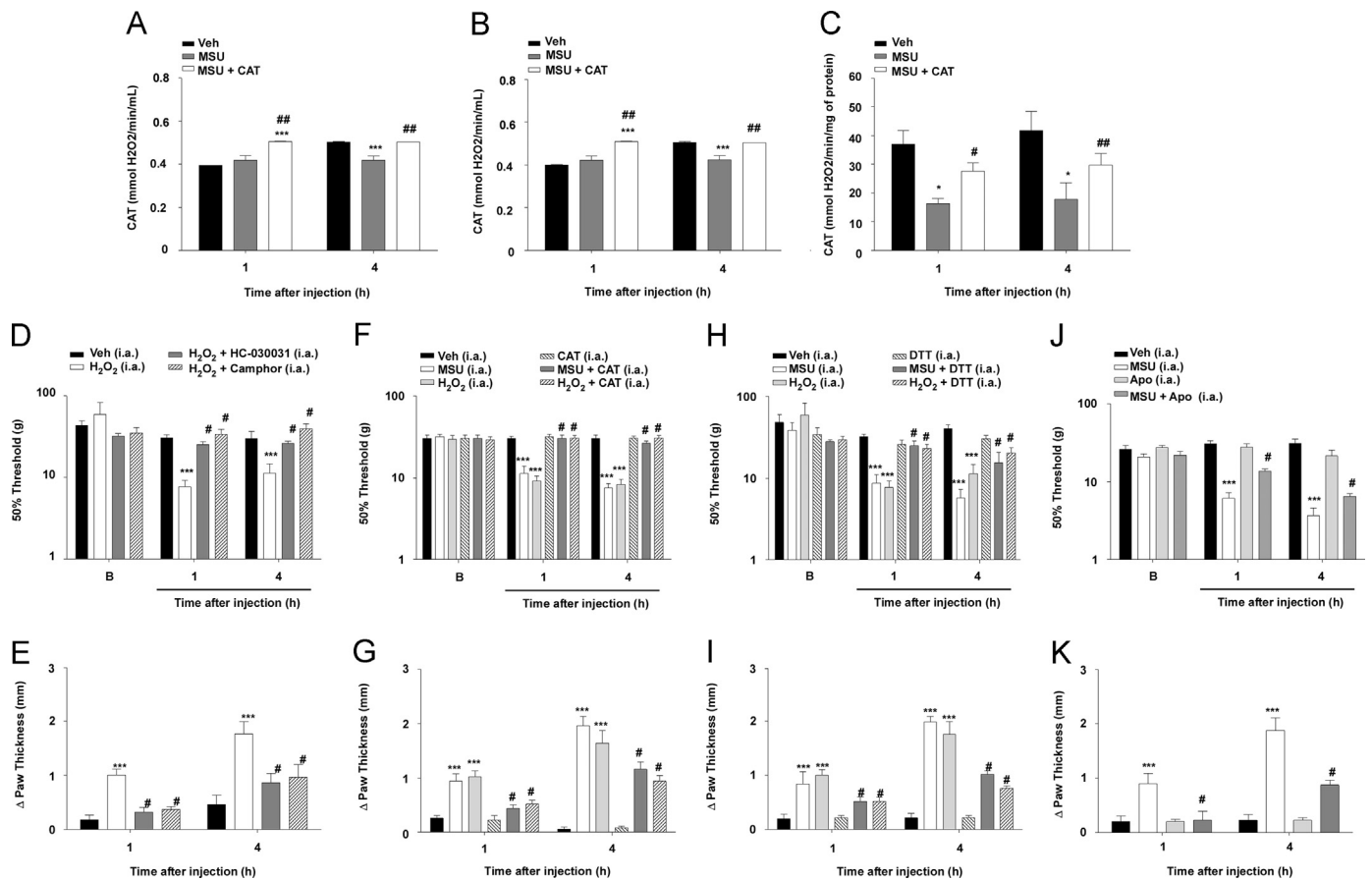


Fig. 4. Monosodium urate crystal-induced nociception and edema were mediated via activation of TRPA1 receptors by hydrogen peroxide. The ia injection of MSU altered the catalase (CAT) activity observed in the synovial fluid (A) pellet and (B) supernatant samples or in the (C) synovial tissue, and the co-injection of CAT (300 UI/site, ia) with MSU (1.25 mg/site, ia) prevented this effect. The selective and nonselective TRPA1 antagonists HC-030031 (300 nmol/site, ia) and camphor (150 nmol/site, ia), respectively, when co-injected with H₂O₂ (3 μmol/site, ia) decreased the (D) mechanical hyperalgesia and (E) edema formation induced by this TRPA1 agonist. The co-injection of CAT (300 UI/site, ia) with H₂O₂ (3 μmol/site, ia) or MSU (1.25 mg/site, ia) crystals reduced the (F) mechanical hyperalgesia and (G) edema formation. The reducing agent DTT (20 nmol/site, ia) was co-injected with MSU (1.25 mg/site, ia) or H₂O₂ (3 μmol/site, ia), and it reduced the (H) mechanical hyperalgesia and (I) edema induced by ia injection of MSU or H₂O₂. The NADPH-oxidase inhibitor apocynin (Apo; 1 μg/site, ia) reduced the (J) hyperalgesia and (K) edema caused by MSU (1.25 mg/site, ia) injection. B represents the baseline threshold of animals. Each column represents the mean ± SEM of six rats. Significance levels: **P* < 0.05, ****P* < 0.001 compared with vehicle (Veh)-treated group; or #*P* < 0.05, ##*P* < 0.05 in comparison to MSU- or H₂O₂-treated group; one-way (A–C) or two-way (in D–K) ANOVA followed by Bonferroni's post hoc test.

Previous findings show that MSU challenge generated oxidative stress from resident cells and neutrophils, and both responses were reduced by colchicine [31,37,38]. Thus, by analogy, we propose that resident synovial cells, which have the potential to phagocytize MSU crystals, generate H₂O₂ when challenged with MSU. In the normal synovial tissue, type A synoviocytes are known to exert a macrophage-like behavior, with the associated phagocytic activity [39–41]. Previously, it has been described that injection of MSU crystals into the synovial space in dogs produced an inflammatory response (marked infiltration of neutrophils) that was initiated by premature MSU crystal phagocytosis by resident cells (synovial lining cells) in the synovial space [42].

Consistent with these findings, ia injection of H₂O₂ evoked nociception and inflammation in a TRPA1-dependent manner. In addition, catalase or the reducing agent DTT decreased these effects. Moreover, we observed that catalase activity was decreased after MSU ia injection in the synovial fluid (supernatant, which represents the extracellular enzyme, and pellet, which represents intracellular enzyme, after 4 h) and also in the synovial tissue (1 and 4 h after MSU injection). In addition, the inhibition of NADPH oxidase by apocynin was able to reduce MSU-induced hyperalgesia, edema, and inflammatory cell infiltration. Then, the increase in NADPH oxidase and the decrease in catalase activities after MSU ia injection may account for the observed H₂O₂

production, which could in turn activate the TRPA1 receptor causing hyperalgesia and inflammation. In this context, it should be emphasized that gout patients show levels of reactive oxygen species higher than those present in normal subjects [3,6].

MSU-mediated inflammation has been linked to IL-1β production, which follows the activation of infiltrating cells [43–45]. Indeed, neutralization of IL-1β has been explored as a new strategy for the relief of gout pain [46,47]. However, the present data indicate that TRPA1 activation, presumably by H₂O₂, is upstream of neutrophil infiltration and increased IL-1β production. In fact, early blockade of TRPA1 receptor or of H₂O₂ production by catalase reduced the MSU-evoked increase in IL-1β, MPO activity, or neutrophil accumulation in the synovial space. Also, CGRP antagonism and NADPH-oxidase blockage reduced the neutrophil accumulation in the synovial fluid.

The increase in TRPA1 expression after MSU injection was delayed and this fact is consistent with the time lag usually required either for new protein generation or for transport to the nerve terminals. The temporal association between the increase in TRPA1 expression and the continuation of the hypersensitivity and inflammatory condition suggests that maintenance rather than onset of these phenomena is dependent on augmented channel expression. It is possible, as observed by others [48], that inflammatory mediators, including IL-1β, promote TRPA1

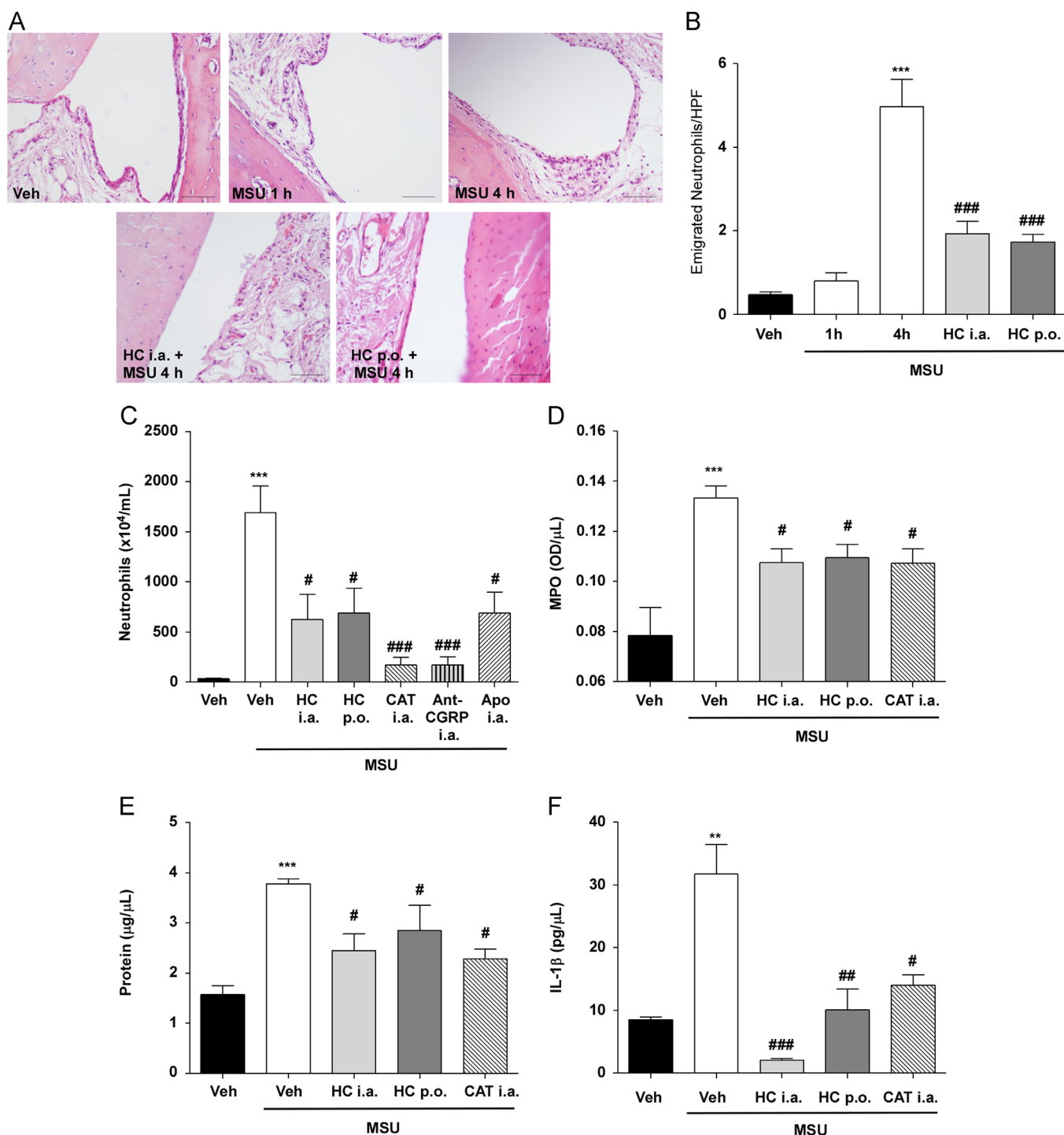


Fig. 5. Monosodium urate crystal-induced inflammatory response is largely reduced by TRPA1 antagonism and blockade of H_2O_2 production. (A) Representative images and (B) pooled data showing the accumulation of emigrated neutrophils induced by the ia injection of MSU (1.25 mg/site) in rats at different time points (1 and 4 h), evaluated by H&E staining and histological analysis. The ia co-injection of MSU with the TRPA1 antagonist HC-030031 (HC, 300 nmol/site) and oral (po) pretreatment with HC (300 μ g/kg, 1 h) significantly reduced the emigration of neutrophils 4 h post-MSU injection. HPF, high power field; original magnification $\times 200$; scale bar, 100 μ m). The ia co-injection of MSU (1.25 mg/site) with HC (300 nmol/site) or catalase (CAT; 300 UJ/site) and oral pretreatment with HC-030031 (300 μ g/kg, po, 1 h) decreased the (C) neutrophil infiltration, (D) MPO activity, (E) plasma extravasation, and (F) IL-1 β levels observed 4 h after ia MSU injection. In addition, (C) the ia co-injection of MSU (1.25 mg/site) with the CGRP receptor antagonist (Ant-CGRP; CGRP 8-37, 1 nmol/site) or apocynin (Apo; 1 μ g/site) decreased the neutrophil infiltration. All the measurements were performed using synovial lavage samples obtained from pretreated rats. Each column represents the mean \pm SEM of six samples. Significance levels: *** P < 0.01, **** P < 0.001 compared with the vehicle (Veh)-treated group; or # P < 0.05, ### P < 0.01, **** P < 0.001 compared with the MSU-injected group; one-way ANOVA followed by Bonferroni's post hoc test.

expression and contribute, by this and other mechanisms, to increased firing of nociceptive neurons and nociception [11,49]. As presented in Fig. 3, TRPA1 is not expressed in leukocytes, indicating that TRPA1 increased expression is not caused by TRPA1

expressed in infiltrating cells. Thus, we speculate that the increase in TRPA1 expression is mediated by local protein synthesis by a resident cell, especially sensory neurons because they may quickly synthesize protein in their axons [50] and their

defunctionalization is largely able to reduce either TRPA1 expression or MSU-triggered inflammation. However, we cannot exclude that the increased expression of TRPA1 could also occur in other cell types present in the synovial cavity, such as synoviocytes [11]. Additional studies must be carried out to clarify the types of cells in which the TRPA1 receptor protein has an increased expression after MSU ia injection.

In conclusion, as previously observed in subcutaneous tissue, our present data support the notion that early TRPA1 activation by endogenous reactive oxygen species in the synovial tissue mediated nociceptive and inflammatory responses induced by MSU ia injection. These findings point to TRPA1 antagonism and the detoxifying of H₂O₂ production by catalase as a possible novel therapeutic option for gout treatment.

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