

## Title page

# TRPA1 mediates aromatase inhibitor-evoked pain by the aromatase substrate androstenedione

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**Abstract:** Aromatase inhibitors (AI) induce painful musculoskeletal symptoms (AIMSS), which are dependent upon the pain transducing receptor TRPA1. However, since the AI concentrations required to engage TRPA1 in mice are higher than those found in the plasma of patients, we hypothesized that additional factors may cooperate to induce AIMSS. Here we report that the aromatase substrate androstenedione, unique among several steroid hormones, targeted TRPA1 in peptidergic primary sensory neurons in rodent and human cells expressing the native or recombinant channel. Androstenedione dramatically lowered the concentration of letrozole required to engage TRPA1. Notably, addition of a minimal dose of androstenedione to physiologically ineffective doses of letrozole and oxidative stress byproducts produces AIMSS-like behaviors and neurogenic inflammatory responses in mice. Elevated androstenedione levels cooperated with low letrozole concentrations and inflammatory mediators were sufficient to provoke AIMSS-like behaviors. The generation of such painful conditions by small quantities of simultaneously-administered TRPA1 agonists justifies previous failure to identify a precise link between aromatase inhibitors and AIMSS, underscoring the potential of channel antagonists to treat AIMSS.

## Introduction

Aromatase inhibitors (AIs) are a mainstay in the treatment of estrogen-sensitive breast cancer in postmenopausal women (1). AIs block the activity of aromatase cytochrome P450, which transforms the androgens, androstenedione and testosterone into the estrogens (estrone and 17 $\beta$ -estradiol, respectively) (2), which are responsible for cancer cell replication and growth (3). Unfortunately, one-third of patients treated with AIs develop muscular and joint pain (aromatase inhibitor-associated musculoskeletal symptoms, AIMSS), a condition that affects the quality of life of patients and limits adherence to AI therapy (4, 5, 6). The underlying mechanism of AIMSS is unknown and, accordingly, the treatment of AIMSS remains an unmet medical need.

Recently, we reported that the transient receptor potential ankyrin 1 (TRPA1), a cation channel highly expressed by a subpopulation of primary sensory neurons of the dorsal root ganglia (DRG) (7), mediates AIMSS-like behaviors evoked by AIs in mice (8). TRPA1-expressing nociceptors contain the neuropeptides substance P (SP) and calcitonin gene-related peptide (CGRP), which mediate neurogenic inflammation (9). Exogenous compounds, including allyl isothiocyanate (AITC), and endogenously generated reactive oxygen species (ROS) and their derivatives, have been identified as TRPA1 agonists (9-12). Similar to other reactive agonists (7), highly electrophilic conjugated Michael acceptor groups of exemestane (13) and nitrile moieties of letrozole and anastrozole (14) react with the thiol groups of specific cysteine and lysine residues to trigger TRPA1 and activate nociceptors (8).

The ability to gate TRPA1 *in vitro* was confirmed *in vivo* by the observation that the pain-like behaviors evoked by AIs in mice are abrogated by genetic deletion or pharmacological blockade of the channel (8). However, AI concentrations required for TRPA1 gating *in vitro* (8) are 1-2 order of magnitude higher than those found in patient plasma (15). In addition, an important proportion (30-40%), but not all, of treated patients develop the painful condition

(16, 17). These observations suggest that exposure to AIs is necessary, but not sufficient, to produce AIMSS, and that additional factors should cooperate with AIs to promote pain symptoms.

Aromatase inhibition, while reducing downstream production of estrogens, moderately increases upstream plasma concentrations of androgens, including androstenedione (ASD) (18). Exemestane, a false aromatase substrate, blocks enzymatic activity by accommodating in the binding pocket that snugly encloses ASD (2). We reasoned that ASD, which retains some of the reactive chemical features of exemestane, such as the  $\alpha,\beta$ -carbonyl moiety of the A ring and the ketone group at the 17 position, might target TRPA1 (Supplementary Fig. S1A). In this paper, we report that ASD, unique among several steroid hormones upstream to aromatase, targets the recombinant and native TRPA1, thereby, alone or in combination with oxidative stress byproducts, contributing to dramatically lowering the concentrations/doses of letrozole required to engage TRPA1 *in vitro* and produce TRPA1-dependent AIMSS-like behaviors in mice.

## **Material and Methods**

### ***Animals***

*In vivo* experiments and tissue collection were carried out according to the European Union (EU) guidelines and Italian legislation (DLgs 26/2014, EU Directive application 2010/63/EU) for animal care procedures, and under University of Florence research permits #204/2012-B and #194/2015-PR. C57BL/6 mice (male, 20-25 g, 5 weeks; Envigo, Milan, Italy; N=284), littermate wild type (*Trpa1*<sup>+/+</sup>, N=22) and TRPA1-deficient (*Trpa1*<sup>-/-</sup>; N=22) mice (25-30 g, 5-8 weeks), generated by heterozygotes on a C57BL/6 background (B6.129P-*Trpa1*<sup>tm1Kykw/J</sup>; Jackson Laboratories, Bar Harbor, ME, USA) (19), TRPV1-deficient mice (*Trpv1*<sup>-/-</sup>; B6.129X1-*Trpv1*<sup>tm1Jul/J</sup>; N=8) backcrossed with C57BL/6 mice (*Trpv1*<sup>+/+</sup>; N=8) for

at least 10 generations (Jackson Laboratories, Bar Harbor, ME, USA; 25-30 g, 5-8 weeks) or Sprague-Dawley rats (male, 75-100 g, Envigo, Milan, Italy; N=24) were used. Animals were housed in a temperature- and humidity-controlled *vivarium* (12-hour dark/light cycle, free access to food and water). Behavioral experiments were done in a quiet, temperature-controlled (20 to 22 °C) room between 9 a.m. and 5 p.m., and were performed by an operator blinded to genotype and drug treatment. Animals were euthanized with inhaled CO<sub>2</sub> plus 10-50% O<sub>2</sub>.

### ***Reagents***

The activating peptide of the human protease activated receptor 2 (hPAR2-AP) was synthesized by G. Cirino (University of Naples, Naples, Italy), and dissolved in distilled water. HC-030031 was synthesized as previously described (20). Letrozole, L-733,060, and CGRP<sub>8-37</sub> were from Tocris Bioscience (Bristol, UK). If not otherwise indicated, all other reagents were from Sigma-Aldrich (Milan, Italy). For *in vitro* experiments, all compounds were dissolved in 100% DMSO at 10 mM concentration.

### ***Cell culture and isolation of primary sensory neurons***

Naive untransfected HEK293 cells (American Type Culture Collection, Manassas, VA, USA; ATCC® CRL-1573™) were cultured according to the manufacturer's instructions. HEK293 cells were transiently transfected with the cDNAs (1 µg) codifying for wild type (wt-hTRPA1) or mutant 3C/K-Q (C619S, C639S, C663S, K708Q) (donated by D. Julius, University of California, San Francisco, CA, USA) (21) human TRPA1 (hTRPA13C/K-Q-HEK293) using the jetPRIME transfection reagent (Euroclone, Milan, Italy) according to the manufacturer's protocol. HEK293 cells stably transfected with cDNA for human TRPA1 (hTRPA1-HEK293, donated by A.H. Morice, University of Hull, Hull, UK), or with cDNA for human TRPV1 (hTRPV1-HEK293, donated by M. J. Gunthorpe, GlaxoSmithKline, Harlow,

UK), or with cDNA for human TRPV4 (hTRPV4-HEK293, donated by N.W. Bunnett, Monash Institute of Pharmaceutical Sciences, Parkville, Australia), or with cDNA for both human TRPA1 and human TRPV1 (hTRPA1/V1-HEK293, hTRPA1/V1-HEK293, donated by A.H. Morice, University of Hull, Hull, UK) (22) were cultured as previously described (22). Human lung fibroblasts (IMR90; American Type Culture Collection, Manassas, VA, USA; ATCC® CCL-186™), which express the native TRPA1 channel, were cultured in DMEM supplemented with 10% FBS, 2 mM glutamine, 100 U penicillin and 100 µg/ml streptomycin. Cells were plated on glass coated (poly-L-lysine, 8.3 µM) coverslips and cultured for 2-3 days before being used for recordings. For all cell lines, the cells were used when received without further authentication. Primary DRG neurons were isolated from adult Sprague-Dawley rats and C57BL/6 or *Trpa1*<sup>+/+</sup> and *Trpa1*<sup>-/-</sup> mice, and cultured as previously described (8).

### ***Cellular Recordings***

Intracellular calcium was measured as previously reported (8). Results are expressed as the percentage of increase of Ratio<sub>340/380</sub> over the baseline normalized to the maximum effect induced by ionomycin (5 µM) (% Change in R<sub>340/380</sub>) or as the percentage of responding neurons, identified by KCl (50 mM). Whole-cell patch-clamp recordings were performed as reported elsewhere (8). Peak currents were normalized to cell membrane capacitance and expressed as mean of the current density (pA/pF) in averaged results. Currents were evoked in the voltage-clamp mode at a holding potential of -60 mV; signals were sampled at 1 kHz and low-pass filtered at 10 kHz. Capsaicin (0.1 or 1 µM) has been used to identify TRPV1-expressing nociceptors.

### ***Behavioral experiments***

For behavioral experiments, after habituation, mice (C57BL/6, *Trpa1*<sup>+/+</sup> and *Trpa1*<sup>-/-</sup>, *Trpv1*<sup>+/+</sup> and *Trpv1*<sup>-/-</sup>) were randomized into treatment groups, consistent with experimental design. First, we evaluated the acute nocifensive response and mechanical allodynia evoked by ASD, H<sub>2</sub>O<sub>2</sub> (20 µl, intraplantar, i.pl.), letrozole (0.5 mg/kg, intragastric, i.g.), or their vehicles [5% DMSO, isotonic saline or 0.5% carboxymethylcellulose (CMC)], respectively. α-lipoic acid (100 mg/kg, i.g.), or its vehicle (0.5% CMC, i.g.), was administered 2 hours after letrozole (i.g.). Mechanical allodynia evoked by the combination of different doses of letrozole (i.g.), ASD (20 µl i.pl.), H<sub>2</sub>O<sub>2</sub> (, 20 µl, i.pl.), or their vehicles was studied in C57BL/6, *Trpa1*<sup>+/+</sup> and *Trpa1*<sup>-/-</sup> mice, with the timing of the various pharmacological interventions based on the timing of the responses to different stimuli. Mechanical allodynia and reduction in grip strength have been studied in C57BL/6 mice, *Trpa1*<sup>+/+</sup> and *Trpa1*<sup>-/-</sup> mice treated with systemic D,L-buthionine sulfoximine (BSO) (intraperitoneal, i.p.), ASD (i.p.) and letrozole (i.g.), or their vehicles (isotonic saline, 4% DMSO and 4% Tween 80 and 0.5% CMC, respectively), alone or in combination, with the timing of the various pharmacological interventions based on the timing of the responses to different stimuli.

*Acute nocifensive response.* Immediately after i.pl. injection of the different compounds, C57BL/6, *Trpa1*<sup>+/+</sup> and *Trpa1*<sup>-/-</sup> mice were placed in a Plexiglas chamber, and the total time spent licking and lifting the injected hind paw was recorded for 5 minutes, as previously described (23).

*Mechanical stimulation (von Frey hair test).* Mechanical threshold was measured in C57BL/6, *Trpa1*<sup>+/+</sup> and *Trpa1*<sup>-/-</sup>, *Trpv1*<sup>+/+</sup> and *Trpv1*<sup>-/-</sup> mice before (basal level threshold) and after the various i.pl. or systemic treatments by using the up-and-down paradigm (24). The 50% mechanical paw withdrawal threshold response (in g) was then calculated as previously described (24, 25).



*Forelimb grip strength test.* The grip strength test was performed with a grip strength meter (Ugo Basile, Varese, Italy), as previously reported (8, 26). The grip strength was measured in C57BL/6, *Trpa1*<sup>+/+</sup> and *Trpa1*<sup>-/-</sup>, *Trpv1*<sup>+/+</sup> and *Trpv1*<sup>-/-</sup> mice before and after the various treatments.

### ***H<sub>2</sub>O<sub>2</sub> levels assay***

Sciatic nerves collected from euthanized C57BL/6 mice after various treatments were homogenized in 50 mM phosphate buffer (pH 7.4) containing 5 mM of sodium azide at 4°C for 60 seconds, centrifuged at 12,000xg for 20 minutes at 4°C, and the supernatant was used to determine the H<sub>2</sub>O<sub>2</sub> content. H<sub>2</sub>O<sub>2</sub> levels were detected by using the phenol red-HRPO method (27, 28) corrected by protein content and expressed as μmol/mg of proteins (29).

### ***Immunostaining***

C57BL/6 mice were anesthetized with a mixture (i.p.) of ketamine (90 mg/kg) and xylazine (3 mg/kg), and transcardially perfused with phosphate buffer saline followed by 4% paraformaldehyde. The sciatic nerve with the surrounding tissue was removed and embedded in paraffin. Immunofluorescence staining for 4-hydroxynonenal adducts and TRPA1 was performed as previously reported (30).

### ***Plasma protein extravasation***

BSO was given 30 minutes before letrozole, and ASD immediately after letrozole; mice were anesthetized 15 minutes after letrozole as previously described (10). HC-030031 (100 mg/kg, i.p.) or its vehicle (4% DMSO plus 4% Tween80, i.p.), or a combination of L-733,060 and CGRP<sub>8-37</sub>, (NK<sub>1</sub>/CGRP-RA; both 2 μmol/kg, i.v.) or its vehicle (isotonic saline), were

administered 60 minutes or 15 minutes before ASD, respectively. The extravasated dye was extracted from synovial tissue of the knee joint by overnight incubation in formamide, and assayed by spectrophotometry at 620 nm, as previously reported (20).

### ***Synovial fluid lavage***

The synovial fluid was collected from anesthetized mice treated with the combination of BSO (i.p.), letrozole (i.g.) and ASD (i.p.), or their vehicles (isotonic saline, 0.5% CMC and 4% DMSO plus 4% Tween80, respectively). BSO was given 30 minutes before letrozole and ASD immediately after letrozole. Synovial fluid was collected by instilling 3 times 0.1 ml of Hank's Buffer plus 10 mM HEPES and 10 mM EDTA in the knee 15 minutes after letrozole. The neutrophil count was performed using standard morphological criteria on Diff-Quick stained cytopins. Data are expressed as total number of neutrophils in 100  $\mu$ l of solution.

### ***CGRP-Like Immunoreactivity assay***

Slices (0.4 mm) of rat spinal cords were superfused with ASD or vehicle. Tissues were pre-exposed to capsaicin (10  $\mu$ M, 20 minutes), superfused with a calcium-free buffer containing EDTA (1 mM), and pretreated with HC-030031 (50  $\mu$ M) or capsazepine (10  $\mu$ M). Superfusate fractions (4 ml) were collected at 10-minute intervals before, during, and after stimulus administration, freeze-dried, reconstituted with assay buffer, and analyzed for CGRP-like immunoreactivity (LI) as previously described (8). CGRP-LI was calculated by subtracting the mean prestimulus value from those obtained during or after stimulation. Results are expressed as femtomoles of peptide per gram of tissue. Stimuli did not cross-react with CGRP antiserum.

### *ASD and letrozole level determination*

ASD levels were measured in mouse serum by using the Active® ASD Radioimmunoassay (Beckman Coulter, CA, USA), a competitive RIA with a sensitivity of 0.1 nmol/L. Radiometric detection was performed using a 2470 WIZARD Automatic Gamma Counter (Perkin Elmer, IL, USA).

Letrozole levels were measured by LC-MS/MS. Briefly, plasma samples (50 µl) were obtained from the blood collected at different time points (1 and 3 hours) after i.g. administration of letrozole (0.1 and 0.5 mg/kg). At 50 µl of plasma sample, 200 pg of d4-letrozole was added. The sample was vortex-mixed for 10 seconds, then 50 µl of ZnSO<sub>4</sub> (90 mg/ml) diluted 1:4 with methanol was added, vortex-mixed for 30 seconds, and centrifuged at 12,000 rpm for 10 minutes. Supernatant (50 µl) was injected in On-line column-switching SPE (CS-SPE). The CS-SPE consists of two high-performance-liquid chromatography (HPLC) systems connected by a six-port switching valve. In the first step, analytes of interest are retained on column 1 (trapping column, SPE Strata C18 20µm, 20 x 2 mm, Phenomenex, Torrance, CA, USA), whereas the matrix components can be washed off. In the second step, column 1 is switched in back-flush to column 2 (analytical column, LUNA, 3 µm, C18 20 x 2 mm, Mercury MS Phenomenex, Torrance, CA, USA). The mobile phases were the same for the trapping column and the analytical column, eluent A water with 0.1% formic acid and eluent B methanol. Samples were measured with a Perkin Elmer Sciex (Thornhill, Canada) API 365 triple quadrupole mass spectrometer equipped with a Turbo IonSpray source, operating in positive ion mode, interfaced with a HPLC Perkin Elmer pump series 200. The capillary voltage was set to 5.5 kV. Heated turbo gas (450° C, air) at a flow rate of 10 l/minutes was used. The ion transitions recorded in Multiple Reaction Monitoring (MRM) were m/z 286.2→217.2 for letrozole and 290.2→221.2 for d4-letrozole. A calibration curve was constructed for letrozole using the appropriate internal standard (d4-letrozole). Plasma samples

(50  $\mu$ l) from control mice were spiked with different concentrations of letrozole (from 2 to 16 ng/ml). A satisfying linearity was obtained for letrozole ( $r^2=0.994$ ).

### ***Data Analysis***

Data represent mean $\pm$ SEM. Statistical analysis was performed by the unpaired two-tailed Student's t-test for comparisons between two groups, and the ANOVA, followed by the Bonferroni *post-hoc* test, for comparisons between multiple groups (GraphPadPrism version 5.00, San Diego, CA, USA). Agonist potency was expressed as half maximal effective concentration ( $EC_{50}$ ); that is, the molar concentration of an agonist producing 50% of the maximum measured effect.  $P<0.05$  was considered statistically significant.

### **Results**

#### ***ASD selectively activates the recombinant and native human TRPA1 by targeting key electrophilic amino acid residues***

In hTRPA1-HEK293 cells, but not in untransfected HEK293 cells, ASD evoked calcium responses in a concentration-dependent manner ( $EC_{50}$ , 49  $\mu$ M) (Fig. 1A and Supplementary Fig. S1B). Responses to both ASD and the TRPA1 agonist, AITC, were abrogated by HC-030031 (Fig. 1B). Consistently, hTRPA1-HEK293 cells superfused with ASD elicited concentration-dependent inward currents (Supplementary Fig. S1E), an effect blocked by HC-030031 (Fig. 1C) and absent in untransfected HEK293 cells (Fig. 1D). In hTRPV1-HEK293 and hTRPV4-HEK293, activated by the selective TRPV1 agonist, capsaicin, or the selective TRPV4 agonist, GSK1016790A, respectively, ASD failed to evoke calcium responses or inward currents (Fig. 1E and 1F). Notably, hTRPA13C/K-Q-HEK293

did not respond to AITC or ASD, while they did respond to the non-electrophilic TRPA1 agonist, menthol (Fig. 1G and Supplementary Fig. S1D) (8).

In IMR90 cells, which constitutively express the TRPA1 channel (31) and do not respond to capsaicin, indicating the absence of a functional TRPV1 channel (Fig. 1H), ASD produced concentration-dependent ( $EC_{50}$ , 37  $\mu$ M) calcium responses that were fully and selectively inhibited by HC-030031 (Fig. 1H and Supplementary Fig. S1C). Similar results were obtained in electrophysiology experiments, where ASD activated TRPA1-mediated inward currents that were entirely and selectively abolished by HC-030031 (Fig. 1I). TRPA1 selectivity of HC-030031 in inhibiting ASD-evoked responses was supported by failure to affect responses produced by hPAR2-AP or KCl in hTRPA1-HEK293 or IMR90 (Fig. 1A-D, 1H, 1I). Next, we wondered whether the other aromatase substrate, testosterone, or steroid hormones upstream to aromatase that maintain the  $\alpha,\beta$ -carbonyl moiety of the A ring (progesterone, 17-hydroxy-progesterone), or the ketone group at the 17 position (dehydroepiandrosterone), or other steroid hormones that retain the  $\alpha,\beta$ -carbonyl moiety of the A ring (aldosterone, cortisol, corticosterone, deoxycorticosterone, 11-deoxycortisol), were able to activate hTRPA1-HEK293 cells. No hormone evoked a measurable response (Supplementary Fig. S1E).

***ASD excites DRG neurons by a prominent role of TRPA1 and, surprisingly, with the contribution of TRPV1***

ASD evoked concentration-dependent ( $EC_{50}$  = 27  $\mu$ M) calcium responses in a subset of rat DRG neurons, identified by their ability to respond to KCl, AITC and capsaicin as nociceptors (23) (Fig. 2A and Supplementary Fig. S1F). Capsaicin-sensitive neurons that did not respond to AITC were also unresponsive to ASD (Fig. 2A). The percentages of ASD-

responding and AITC-responding neurons out of the KCl-responding neurons were similar (Fig. 2B). Superimposable findings were obtained by electrophysiological recording (Fig. 3A). Surprisingly, the remarkable ASD selectivity for TRPA1 was challenged by experiments in rat DRG neurons. The selective TRPV1 antagonist, capsazepine, reduced both the calcium response and the inward currents evoked by ASD, and it abated the residual response observed in the presence of HC-030031 (Fig. 2C and 2D and Fig. 3A). The calcium response to ASD was, however, unaffected by the selective TRPV4 antagonist, HC-067047 (Fig. 2D). Results obtained in rat DRG neurons were replicated in mouse DRG neurons. Cells obtained from *Trpa1*<sup>-/-</sup> mice exhibited a residual calcium response to ASD that, being consistently unaffected by HC-030031, was abated by capsazepine (Fig. 2E). The percentages of ASD-responding and AITC-responding neurons out of the KCl-responding neurons were similar (Fig. 2F) in DRG neurons isolated from *Trpa1*<sup>+/+</sup> mice. The percentage of neurons from *Trpa1*<sup>-/-</sup> mice that exhibited a residual calcium response to ASD did not exceed the percentage obtained in neurons from *Trpa1*<sup>+/+</sup> mice (Fig. 2F).

To confirm the contribution of TRPV1 in the overall response to ASD, we used hTRPA1/V1-HEK293 cells (22), where the calcium response evoked by ASD was reduced by both HC-030031 and capsazepine, and was abated solely by the combination of the two antagonists, while responses to AITC and capsaicin were fully attenuated by respective antagonists (Fig. 2G). Thus, TRPV1, when co-expressed with TRPA1, as constitutively happens in DRG neurons, appears to contribute to the response to ASD.

CGRP release from slices of the rat dorsal spinal cord, an anatomical site enriched with terminals of TRPA1-positive peptidergic nociceptors, illustrates the ability of ASD to activate such neurons (8). The increased CGRP-LI outflow by ASD was abated by the removal of extracellular calcium, previous desensitization to capsaicin, or in the presence of HC-030031,

but only partially reduced by capsazepine (Fig. 2H). Thus, ASD elicits CGRP release from a subset of TRPV1-positive neurons *via* a neurosecretory process, mediated by TRPA1.

### ***ASD cooperates with letrozole and H<sub>2</sub>O<sub>2</sub> to excite nociceptors in vitro***

To explore whether ASD cooperates with AIs and proinflammatory mediators to excite nociceptors *via* a TRPA1-dependent final common pathway, we used letrozole, the most prescribed AI in clinical practice (32). As a prototypical proinflammatory mediator, we selected the ROS H<sub>2</sub>O<sub>2</sub>, because oxidative stress is increased by breast cancer (33) and letrozole (34). In addition, the effect of letrozole (0.5 mg/kg, i.g.), at a dose that was previously shown to produce *per se* mechanical allodynia, was partially reduced by the antioxidant,  $\alpha$ -lipoic acid (Supplementary Fig. S2). These observations suggest that the TRPA1-dependent letrozole-evoked mechanical hypersensitivity (8) is partially due to ROS generation, which cooperates with the anticancer drug to target TRPA1.

From the concentration-response curves of ASD, letrozole, and H<sub>2</sub>O<sub>2</sub> (Fig. 3A-C), we selected subthreshold concentrations that were unable to elicit measurable inward currents in rat DRG neurons, and combined a *per se* inactive concentration of ASD with inactive concentrations of letrozole or H<sub>2</sub>O<sub>2</sub> (ASD/letrozole or ASD/H<sub>2</sub>O<sub>2</sub>). Interestingly, we found that each combination evoked inward currents, which were abated by TRPA1 antagonism and reduced by TRPV1 antagonism (Fig. 3D). Finally, we identified an inactive combination of letrozole and H<sub>2</sub>O<sub>2</sub> (Fig. 3E), and we found that adding an inactive ASD concentration to it triggered an inward current that was abated by HC-030031 and only partially reduced by capsazepine (Fig. 3E).

***ASD cooperates with letrozole and H<sub>2</sub>O<sub>2</sub> to produce local TRPA1-dependent mechanical allodynia***

Previous *in vitro* findings were translated to an *in vivo* setting. Injection of ASD (1-10 nmol/paw) into the mouse paw did not evoke any acute nociceptive behavior (data not shown). However, 30 minutes after the injection, and for the following 2 hours, ASD produced a dose-dependent mechanical allodynia (Fig. 4A and 4B) that was partially and completely abrogated by capsazepine and HC-030031, respectively (Fig. 4B). H<sub>2</sub>O<sub>2</sub> injected in the mouse paw produced a dose-dependent mechanical allodynia that was entirely dependent on TRPA1 (Fig. 4C). Similar to previous findings (8), systemic letrozole (0.1-0.5 mg/kg, i.g.) evoked a dose-dependent, delayed (1-6 hours) mechanical allodynia that was abated by HC-030031 and unaffected by capsazepine (Fig. 4D). The combined administration of allodynia-evoking doses of ASD and letrozole produced an exaggerated pain-like response (Supplementary Fig. S3A).

Next, we found doses of ASD and letrozole, or H<sub>2</sub>O<sub>2</sub> and ASD, which, although *per se* ineffective, when given in combination, lowered the threshold for eliciting mechanical allodynia (Supplementary Fig. S3B and S3C). Finally, we identified ineffective combinations of ASD/letrozole, letrozole/H<sub>2</sub>O<sub>2</sub>, or H<sub>2</sub>O<sub>2</sub>/ASD that, when given simultaneously (letrozole/ASD/H<sub>2</sub>O<sub>2</sub>), caused mechanical allodynia (Fig. 4E). This response was partially reduced by capsazepine, completely reverted by HC-030031 (Fig. 4F), and absent in *Trpa1*<sup>-/-</sup> (Fig. 4G).

***ASD cooperates with letrozole and H<sub>2</sub>O<sub>2</sub> to produce systemic TRPA1-dependent AIMSS-like behaviors and neurogenic inflammation***

In mice, letrozole (0.5 mg/kg, i.g.) has been reported to evoke TRPA1-dependent mechanical allodynia and a decrease in grip strength, two effects reminiscent of AIMSS (8).



The same dose of letrozole (0.5 mg/kg, i.g.) (8) increased H<sub>2</sub>O<sub>2</sub> in the sciatic nerve tissue and slightly augmented ASD serum levels (Fig. 5A and 5C). BSO, by inhibiting  $\gamma$ -glutamylcysteine synthetase, causes systemic depletion of glutathione, and the ensuing increase in ROS (35). BSO (800 mg/kg, i.p.) increased mechanical allodynia and decreased forelimb grip strength through a TRPA1-dependent mechanism (Supplementary Fig. S4A), while, at the dose of 400 mg/kg, it slightly increased H<sub>2</sub>O<sub>2</sub> in the sciatic nerve tissue (Fig. 5C) without affecting pain-like behaviors (Supplementary Fig. S4A). Finally, systemic administration of ASD (2  $\mu$ g/kg, i.p.) caused, in mice, mechanical allodynia and reduced forelimb grip strength *via* TRPA1, with a partial contribution of TRPV1 (Supplementary Fig. S4B). ASD (2  $\mu$ g/kg, i.p.) also increased H<sub>2</sub>O<sub>2</sub> levels in the sciatic nerve (Fig. 5C).

To better understand the contribution of ASD and oxidative stress to the AIMSS-like behaviors, a low dose of ASD (0.2  $\mu$ g/kg, i.p.) that failed to affect H<sub>2</sub>O<sub>2</sub> generation (Fig. 5C), as well as mechanical allodynia and forelimb grip strength, (Supplementary Fig. S4B) was used. This same dose slightly increased hormone plasma concentration to levels comparable to those produced by a dose of letrozole (0.5 mg/kg, i.g.) that caused pain-like behaviors (Fig. 5A and Fig. 4D). In mice, 1 hour after oral administration of letrozole (0.1 and 0.5 mg/kg), drug concentrations in plasma were  $13.5 \pm 2.0$  ng/ml (n = 4) and  $55.3 \pm 4.8$  ng/ml (n = 4), respectively, whereas 3 hours after dosing, plasma concentrations were  $8.41 \pm 1.0$  ng/ml (n = 4) and  $45.4 \pm 6.11$  ng/ml (n = 4), respectively. Notably, the plasma concentration of 0.5 mg/kg letrozole measured at 1 hour after dosing was similar to that found previously (8). Systemic BSO (400 mg/kg, i.p.), letrozole (0.1 mg/kg, i.g.) and ASD (0.2  $\mu$ g/kg, i.p.), which *per se*, or in combinations (letrozole/ASD, BSO/ASD or BSO/letrozole), did not affect behavioral responses, when given simultaneously caused remarkable mechanical allodynia and decreased forelimb grip strength (Fig. 5D and 5E). The triple combination of BSO, letrozole, and ASD increased H<sub>2</sub>O<sub>2</sub> levels in the sciatic nerve (Fig. 5C). However, the increase equals that evoked

by BSO (400 mg/kg) alone. Finally, the remarkable increase in 4-hydroxynonenal staining in the sciatic nerve allowed us to localize the oxidative stress generation within the neural structure (Fig. 5H). Behavioral responses evoked by the triple combination were partially and totally reverted by capsazepine and HC-030031, respectively (Fig. 5D and 5E), and were absent in *Trpa1*<sup>-/-</sup> mice, but unaffected in *Trpv1*<sup>-/-</sup> mice (Fig. 5F and 5G).

Finally, the combination of BSO (400 mg/kg, i.p.), letrozole (0.1 mg/kg, i.g.) and ASD (0.2 µg/kg, i.p.), which produced AIMSS-like behaviors, increased Evans blue dye extravasation in the synovial tissue and the number of neutrophils in the synovial fluid of mouse knee joint (Fig. 5I and 5J). Both responses were reduced by pretreatment with HC-030031 or a combination of L733,060 and CGRP<sub>8-37</sub> (both 2 µmol/kg, i.v.) (Fig. 5I and 5J). These findings indicate that the letrozole/ASD/BSO combination *via* TRPA1 promotes two typical neurogenic inflammatory responses, such as plasma protein and neutrophil extravasation (9).

## Discussion

We found that ASD, unique among several steroid hormones, activates TRPA1, thereby promoting AIMSS-like responses. ASD behaves as a TRPA1 agonist across species, as it engages both the recombinant and native human channel and the rat and mouse channel. TRPA1 activation by ASD, similar to other electrophilic agonists (7), requires the presence of three cysteine (C619, C639, C663) and one lysine (K708) key residues. Furthermore, ASD exhibits a peculiar selectivity profile. Whereas in cells expressing only one channel (transfected HEK293 cells, IMR90 human fibroblasts) (31, 36), ASD is a selective TRPA1 agonist, surprisingly, in rodent DRG neurons, which express multiple TRP channels, TRPV1 contributes to TRPA1-dependent ASD-evoked responses. Notably, DRG neurons from *Trpa1*<sup>-/-</sup> mice maintained a residual responsiveness to ASD which was abolished by capsazepine. One possible explanation for this unexpected finding is that the TRPA1 protein remaining after the

homologous recombination, while lacking the domain required for channel activation by most agonists (37), including ASD itself, maintains the domain essential for TRPV1-dependent ASD activity. TRPV1 contribution to TRPA1-mediated responses evoked by ASD or other chemicals (22) was confirmed in cells expressing the recombinant forms of both the TRPA1 and TRPV1 (hTRPA1/V1-HEK293) channels. Formation of heterotetramers (38, 39), or intracellular calcium movement initiated by TRPA1 gating that results in a secondary TRPV1 activation (40), may explain how ASD heterologously interregulates channel activities (41, 42). Notwithstanding, this peculiar selectivity pattern was not replicated *in vivo* as TRPA1 inhibition was sufficient to totally prevent ASD actions.

Tenosynovitis and joint swelling are symptoms reported by patients treated with AIs (43). However, association with proinflammatory markers, including cytokines, such as interleukin-6, has been excluded (44). The ability of ASD to release sensory neuropeptides or, in combination with letrozole and BSO, to provoke TRPA1-dependent SP/CGRP release, edema and neutrophil infiltration in the knee joint, suggests that neurogenic inflammation mediates the inflammatory component of AIMSS. The recent observation that, due to their electrophilic and reactive properties, exemestane, anastrozole and letrozole target TRPA1, thus evoking pain-like responses and neurogenic inflammation, supported the hypothesis that channel activation in peptidergic nociceptors promotes AIMSS (8). However, while the three AIs target TRPA1 with remarkable selectivity, they exhibit low potency at both human and rodent channels (8). Notably, the peak plasma concentration of letrozole measured in mice in the present study after the 0.5 mg/kg dose (~194 nM) is close to the maximum concentration reported in human plasma after a single therapeutic dose (2.5 mg) (~128 nM) (45). It must be underlined that the plasma concentrations found in mice and humans are 50-400 times lower than the threshold concentrations of letrozole able to gate *in vitro* the mouse, rat or human TRPA1 (>10  $\mu$ M). This gap between plasma levels and threshold concentrations at TRPA1 argues against the

hypothesis that AIs *per se* cause AIMSS. Elevated tissue concentrations due to high AI volumes of distribution (46) may still be insufficient for effective channel gating. Lack of evidence associating increased AI plasma levels with pain symptoms (45) further weakens the hypothesis that AIMSS results from an exclusive AI action. Similarly, attempts to associate serum ASD concentrations with AIMSS have failed (18). Thus, a direct cause-and-effect relationship between plasma levels of AIs or ASD and AIMSS has not been shown.

TRPA1 is amenable to sensitization by a variety of endogenous proinflammatory and proalgesic mediators (47), and we previously reported that H<sub>2</sub>O<sub>2</sub> or PAR2 stimulation exaggerated TRPA1-dependent responses evoked by AIs (8). The present novel finding that the aromatase substrate, ASD, activates TRPA1 proposes a novel paradigm to explain AIMSS generation. Multiple factors, concomitantly occurring in breast cancer patients treated with AIs, may cooperate to engage TRPA1, thus causing AIMSS-like behaviors. Letrozole (and probably other AIs) is the essential, although *per se* ineffective, initiating stimulus. Letrozole slightly augments ROS (34) and ASD concentrations (18). Breast cancer (33) or incidental inflammatory processes (48) may boost oxidative stress, thus increasing the possibility of the simultaneous presence of the three TRPA1 stimulants. However, the current hypothesis does not exclude that additional agents, able to activate or sensitize the TRPA1, may act along with AIs and ASD to reach the threshold for AIMSS generation. The present study, while showing that TRPV1 signaling negligibly contributes to ASD-evoked AIMSS-like behaviors, robustly underscores the paramount role of TRPA1. Thus, TRPA1 blockade by both new compounds currently under clinical scrutiny and old medicines recently identified as TRPA1 antagonists (49) may represent a new frontier to treat or prevent AIMSS.

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## Figure Legends

**Figure 1.** ASD selectively activates the human TRPA1 channel. A, calcium response evoked by ASD (100  $\mu$ M), AITC (5  $\mu$ M) and hPAR2-AP (100  $\mu$ M) in hTRPA1-HEK293 and in HEK293 cells. B, HC-030031 (HC03; 30  $\mu$ M) abates the response to both ASD and AITC, but not to hPAR2-AP. C and D, ASD (50  $\mu$ M) or AITC (100  $\mu$ M), elicit inward currents in hTRPA1-HEK293, but not in HEK293 cells. HC03 (50  $\mu$ M) does not affect responses to KCl (50 mM), but abolishes responses to either ASD or AITC. E and F, ASD (100  $\mu$ M) is ineffective in hTRPV1-HEK293, activated by capsaicin (CPS; 0.1  $\mu$ M), and in hTRPV4-HEK293, activated by GSK1016790A (GSK; 0.1  $\mu$ M). G, hTRPA1 3C/K-Q HEK293 are insensitive to ASD (100  $\mu$ M), but respond to menthol (100  $\mu$ M), whereas wt-hTRPA1 respond to both compounds. H, IMR90 respond to ASD (100  $\mu$ M) and AITC (1  $\mu$ M), but not to CPS (5  $\mu$ M). Responses to AITC and ASD, but not to hPAR2-AP (100  $\mu$ M), are inhibited by HC03 (30  $\mu$ M), but not by capsazepine (CPZ, 10  $\mu$ M). I, In IMR90 fibroblasts ASD (100  $\mu$ M) and AITC (100  $\mu$ M), but not hPAR2-AP (100  $\mu$ M), evoke inward currents, which are abated by HC03 (50  $\mu$ M). Veh is the vehicle of ASD; (-) indicates the vehicle of antagonists. Each point/column is the mean $\pm$ SEM of at least n=25 cells from 3-6 independent experiments for calcium recordings or of at least n=6 cells from 4-8 independent experiments for electrophysiological recordings. \*P<0.05 vs. Veh, §P<0.05 vs. ASD or AITC. ANOVA and Bonferroni *post hoc* test.

**Figure 2.** ASD activates the native TRPA1 channel expressed in rodent DRG neurons. A, typical tracings of calcium responses in rat DRG neurons that respond to ASD (100  $\mu$ M), AITC (30  $\mu$ M) and capsaicin (CPS; 0.1  $\mu$ M) (black line), or solely to CPS (red line). B, percentage of DRG neurons (sensitive to 50 mM KCl) that respond to ASD, AITC or CPS. C and D, in rat DRG neurons concentrations of HC-030031 (HC03; 30  $\mu$ M) and capsazepine (CPZ; 10  $\mu$ M)

that selectively and completely attenuated AITC and CPS responses, respectively, partially inhibit the response to ASD that, however, is abated by their combination (HC03/CPZ). HC-067047 (HC06; 10  $\mu$ M) does not affect the response to ASD. E, ASD produces a calcium response in CPS-sensitive DRG neurons isolated from *Trpa1*<sup>+/+</sup> mice. The residual response to ASD in neurons from *Trpa1*<sup>-/-</sup> mice is abated by CPZ. F, percentage of *Trpa1*<sup>+/+</sup> or *Trpa1*<sup>-/-</sup> DRG neurons responsive to AITC, ASD and CPS. G, in hTRPA1/V1-HEK293 cells the calcium response to ASD is partially inhibited by HC03 or CPZ and abated by their combination (HC03/CPZ). Calcium responses to AITC (5  $\mu$ M) and CPS (1  $\mu$ M) are abolished by HC03 and CPZ, respectively. Each point/column represents the mean $\pm$ SEM of at least n=25 neurons from 3-7 independent experiments. \*P<0.05 vs. Veh or Veh-*Trpa1*<sup>+/+</sup>, §P<0.05 vs. ASD, CPS, AITC or ASD-*Trpa1*<sup>+/+</sup>, #P<0.05 vs. ASD-*Trpa1*<sup>-/-</sup>. H, CGRP-LI outflow elicited by ASD (10-50  $\mu$ M) from rat dorsal spinal cord slices is prevented by pre-exposure to CPS (10  $\mu$ M, 20 min; CPS-des) or by calcium removal (Ca<sup>2+</sup>-free) and is attenuated by HC03 (50  $\mu$ M) and only partially reduced by CPZ (10  $\mu$ M). Each column represents the mean $\pm$ SEM of at least 4 independent experiments running in duplicate. \*P<0.05 vs. Veh, §P<0.05 vs. ASD 50  $\mu$ M, #P<0.05 vs. HC03. Veh is the vehicle of ASD; (-) indicates the vehicle of antagonists. ANOVA and Bonferroni *post hoc* test.

**Figure 3.** ASD potentiates TRPA1-mediated inward currents in the presence of subthreshold letrozole and/or proinflammatory stimuli in rat DRG neurons. A, ASD and AITC (100  $\mu$ M) evoke inward currents (whole-cell patch-clamp recordings) in capsaicin (CPS; 1  $\mu$ M)-sensitive rat DRG neurons. ASD-evoked currents are partially reduced by HC-030031 (HC03; 50  $\mu$ M) or capsazepine (CPZ; 10  $\mu$ M), which abated currents evoked by AITC or CPS, respectively. The combination of HC03 and CPZ abated the ASD-evoked currents. B and C, letrozole (LTZ) and H<sub>2</sub>O<sub>2</sub> evoke concentration-dependent inward currents that are abolished by HC03 (50  $\mu$ M).

D, inward currents elicited by a combination of ineffective concentrations of ASD (10  $\mu$ M)/LTZ (50  $\mu$ M) or ASD (10  $\mu$ M)/H<sub>2</sub>O<sub>2</sub> (50  $\mu$ M) are abolished by HC03 and reduced by CPZ. The combination with a lower concentration of ASD (1  $\mu$ M) is ineffective. E, addition of a much lower concentration of ASD (30 nM) to the ineffective combination of LTZ (50  $\mu$ M)/H<sub>2</sub>O<sub>2</sub> (50  $\mu$ M) elicits inward currents, which are abated by HC03 and reduced by CPZ. Veh is the vehicle of ASD, LTZ or H<sub>2</sub>O<sub>2</sub>; (-) indicates the vehicle of antagonists. Results are mean $\pm$ SEM of at least 4 independent experiments. \**P*<0.05 vs. Veh; §*P*<0.05 vs. LTZ, H<sub>2</sub>O<sub>2</sub>, ASD. ANOVA and Bonferroni *post hoc* test.

**Figure 4.** ASD cooperates with letrozole and H<sub>2</sub>O<sub>2</sub> to produce TRPA1-dependent local mechanical allodynia. A, diagram illustrating the treatment schedule before behavioral tests. B-D, In C57BL/6 mice, injection (20  $\mu$ l) of ASD or H<sub>2</sub>O<sub>2</sub> and administration of LTZ induce a dose and time-dependent mechanical allodynia that is reversed completely by HC03, and partially by CPZ. E and F, The combination of ineffective doses of LTZ, ASD and H<sub>2</sub>O<sub>2</sub> evokes mechanical allodynia that is completely prevented by HC03, partially reduced by CPZ, and (G) absent in *Trpa1*<sup>-/-</sup> mice. BL, baseline threshold. VehASD, VehLTZ and VehH<sub>2</sub>O<sub>2</sub> are the vehicle of ASD, LTZ and H<sub>2</sub>O<sub>2</sub>, respectively; (-) is the vehicle of antagonists. Results are mean  $\pm$  SEM of at least n=5 mice for each group. \**P*<0.05 vs. BL; §*P*<0.05 vs. (-); #*P*<0.05 vs. *Trpa1*<sup>+/+</sup>. ANOVA and Bonferroni *post hoc* test.

**Figure 5.** Androstenedione cooperates with letrozole and H<sub>2</sub>O<sub>2</sub> to evoke systemic TRPA1-dependent AIMSS-like behaviors. A, ASD serum levels are similarly increased by systemic administration ( $\downarrow$ ) of ASD or letrozole (LTZ). B, diagram illustrating the treatment schedule before behavioral tests. C, LTZ or ASD increase H<sub>2</sub>O<sub>2</sub> in homogenates of mouse sciatic nerve. Addition of ineffective doses of LTZ and ASD does not further increase H<sub>2</sub>O<sub>2</sub> levels produced by BSO alone. D and E, ASD, LTZ and BSO alone or in dual combinations (ASD/LTZ;

ASD/BSO; LTZ/BSO) do not increase mechanical allodynia or decrease grip strength. However, their combination (BSO/LTZ/ASD) increases mechanical allodynia and decreases grip strength. Both responses are reverted by HC03 and attenuated by CPZ. F and G, changes in mechanical allodynia and grip strength induced by BSO/LTZ/ASD observed in *Trpa1<sup>+/+</sup>* mice are similar to those observed in *Trpv1<sup>+/+</sup>* and abrogated in *Trpa1<sup>-/-</sup>* mice, but not in *Trpv1<sup>-/-</sup>* mice. H, BSO/LTZ/ASD (+++), but not their vehicles (---) increase 4-hydroxynonenal (4-HNE) staining within the sciatic nerve. I and J, HC03 or the combination of L-733,060 and CGRP<sub>8-37</sub> (NK1/CGRP-RA) attenuate increases in Evans blue dye extravasation in the knee joint synovial tissue, and in neutrophil number in synovial fluid evoked by BSO/LTZ/ASD. BL, baseline threshold. Scale bar, 100  $\mu$ m. Veh is the vehicle of LTZ, ASD and BSO; VehASD, VehLTZ and VehBSO are the vehicle of ASD, LTZ and BSO, respectively; VehHC03/CPZ (D) and (-) (I, J) are the vehicle of antagonists. Results are mean $\pm$ SEM of at least n=5 mice for each group. \*P<0.05 vs. Veh (B), time 0 (C) or white columns or circle (D-J); §P<0.05 vs. Veh HC03/CPZ; #P<0.05 vs. *Trpa1<sup>+/+</sup>*; †P<0.05 vs. (-). ANOVA and Bonferroni *post hoc* test.