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# Monosodium iodoacetate-induced inflammation and joint pain are reduced in TRPA1 deficient mice – potential role of TRPA1 in osteoarthritis

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SUMMARY

*Objectives:* Intra-articularly injected monosodium iodoacetate (MIA) induces joint pathology mimicking osteoarthritis (OA) and it is a widely used experimental model of OA. MIA induces acute inflammation, cartilage degradation and joint pain. Transient Receptor Potential Ankyrin 1 (TRPA1) is an ion channel known to mediate nociception and neurogenic inflammation. Here, we tested the hypothesis that TRPA1 would be involved in the development of MIA-induced acute inflammation, cartilage changes and joint pain.

*Methods:* The effects of pharmacological blockade (by TCS 5861528) and genetic depletion of TRPA1 were studied in MIA-induced acute paw inflammation. Cartilage changes (histological scoring) and joint pain (weight-bearing test) in MIA-induced experimental OA were compared between wild type and TRPA1 deficient mice. The effects of MIA were also studied in primary human OA chondrocytes and in mouse cartilage.

*Results:* MIA evoked acute inflammation, degenerative cartilage changes and joint pain in wild type mice. Interestingly, these responses were attenuated in TRPA1 deficient animals. MIA-induced paw inflammation was associated with increased tissue levels of substance P; and the inflammatory edema was reduced by pretreatment with catalase, with the TRPA1 antagonist TCS 5861528 and with the neurokinin 1 receptor antagonist L703,606. In chondrocytes, MIA enhanced interleukin-1 induced cyclooxygenase-2 (COX-2) expression, an effect that was blunted by pharmacological inhibition and genetic depletion of TRPA1.

*Conclusions:* TRPA1 was found to mediate acute inflammation and the development of degenerative cartilage changes and joint pain in MIA-induced experimental OA in the mouse. The results reveal TRPA1 as a potential mediator and drug target in OA.



## Introduction

Osteoarthritis (OA) is the most common joint disease worldwide and its prevalence is increasing as the population ages. The disease is characterized by the degradation of the articular cartilage which is associated with variable joint inflammation and manifests in pain and loss of joint function and eventually in the need for joint replacement surgery.[1,2]

Injection of monosodium iodoacetate (MIA) into the articular cavity induces a joint pathology mimicking that seen in human OA and it has widely been used as an experimental model of OA[3]. MIA induces a local acute inflammation[4,5] which is followed by the development of degenerative changes in the articular cartilage, hyperalgesia and decreased weight-bearing on the affected limb indicative of joint pain[3,6]. Similar to the situation in OA, the detailed mechanisms related to the onset and mediation of MIA-induced experimental OA have not been thoroughly elucidated. At the

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cellular level, MIA has been recognized as an inhibitor of glyceraldehyde-3-phosphate dehydrogenase which induces the production of reactive oxygen species (ROS) and caspase activation[7].

Transient receptor potential ankyrin 1 (TRPA1), an ion channel involved in nociception and neurogenic inflammation, has lately attracted considerable interest as a potential drug target to treat painful and inflammatory conditions[8–10]. In various *in vivo* models, TRPA1 activation has proven to be crucial in the triggering of inflammatory edema[11], hyperalgesia[12] and pain[13,14]. Activation of membrane associated TRPA1 causes an influx of cations, especially $Ca^{2+}$, which mediate the cellular responses of activation of TRPA1 such as nociception and release of proinflammatory neuropeptides substance P, calcitonin gene related peptide and neurokinin A[9]. TRPA1 is ligand-gated channel and its physiological role is believed to act as a chemosensor for environmental potentially noxious compounds such as allyl isothiocyanate (AITC) which is the pungent compound present in mustard oil and widely used as a research tool to activate TRPA1[10,15]. In addition to environmental irritants, many endogenously formed proinflammatory factors such as hydrogen peroxide ($H_2O_2$)[16], nitric oxide[17] and nitro-oleic acid[18] have been demonstrated to activate TRPA1.

Based on the evidence that MIA induces the production of ROS and that ROS are some of the most potent endogenous TRPA1 activators, we postulated that TRPA1 activation could be involved in the development of inflammation and inflammatory pain in the MIA-induced experimental OA. Previously, two reports have been published examining the role of TRPA1 in MIA-induced OA. In those studies, a single dose of a TRPA1 antagonist failed to alter pain-like behaviour or to shift weight-bearing[6] although the treatment was effective in blocking mechanically induced hypersensitivity[19]. However, we hypothesized that TRPA1 could play a role in MIA-induced acute inflammation and therefore contribute to the development of MIA-induced arthritis. In the present study, we utilized both pharmacological blockade and genetic depletion of TRPA1 in order to investigate whether TRPA1 activation could enhance/mediate MIA-induced acute inflammatory edema and cyclooxygenase-2 (COX-2) expression, and development of cartilage changes and joint pain characteristic for MIA-induced experimental OA.

## Materials and methods

### *Animals*

Wild type and TRPA1 knock-out male B6; 129P-Trpa1(tm1-Kykw)/J mice (Charles River Laboratories, Sulzfeld, Germany) were used in the experiments investigating the effects of genetic depletion of TRPA1. Wild type male C57BL/6N mice (Scanbur Research A/S, Karlslunde, Denmark) were used to study the effects of the drugs. Mice were housed under standard conditions (12–12 h light–dark cycle, 22 ± 1°C) with food and water provided *ad libitum*. Animal experiments were carried out in accordance with the legislation for the protection of animals used for scientific purposes (Directive 2010/63/EU) and approved by the National Animal Experiment Board. Intraperitoneal injections of medetomidine (0.5 mg/kg, Domitor®, Orion Oyj, Espoo, Finland) and ketamine (75 mg/kg, Ketalar®, Pfizer Oy Animal Health, Helsinki, Finland) were used to achieve anaesthesia. After the experiments, the animals were sacrificed by carbon monoxide followed by cranial dislocation. Reagents were purchased from Sigma Chemical Co., St. Louis, MO, USA unless otherwise indicated.

### *Studied animal groups*

Wild type mice were dosed with the selective TRPA1 antagonist TCS 5861528 (10 mg/kg perorally), the anti-inflammatory steroid dexamethasone (2 mg/kg intraperitoneally), the hydrogen peroxide decomposing enzyme catalase (300 IU/paw intraplantarly), the selective neurokinin 1 receptor antagonist L703,606 (10 mg/kg intraperitoneally, Enzo Life Sciences AG, Lausen, Switzerland) which has been shown to block the actions of the neuropeptide substance P[20] or with the vehicle (PBS intraperitoneally) prior to the experiments. Orally administered drugs were diluted in 75% polyethylene glycol and given by gavage in a volume of 250 μl 2 h prior to the experiments. Intraperitoneally dosed drugs were diluted in PBS in a volume of 450 μl and given 1 h prior to the experiments. Intraplantarly dosed drugs were diluted in PBS and given simultaneously with the MIA. In addition, the effects of genetic depletion of TRPA1 were studied by comparing responses in TRPA1 knock-out mice and their corresponding wild type counterparts.

### *Inflammatory paw edema test*

Inflammatory paw edema was induced by injecting MIA (400 μg) diluted in 50 μl of sterile 0.9% NaCl intraplantarly into the hind paw of anesthetized mice. The contralateral paw was injected with the vehicle and developed no measurable edema. The paw volume was measured for up to 6 h with a plethysmometer (Ugo Basile, Comerio, Italy) and compared to the baseline value.

### *Paw tissue extraction and substance P measurements*

After the mice had been sacrificed, the inflamed subcutaneous paw tissue which had been injected with MIA and the contralateral subcutaneous paw tissue were collected for analysis into a buffer containing Tris (50 mM, pH 7.4), NaCl (150 mM), 0.5% Triton-X and protease and phosphatase inhibitors phenylmethylsulfonyl fluoride (0.5 mM), sodium orthovanadate (2 mM), leupeptin (0.10 μg/ml), aprotinin (0.25 μg/ml) and NaF (1.25 mM). The tissue was minced and incubated in the lysis buffer for 20 min with constant and vigorous shaking. The samples were centrifuged at 10,000*g* for 10 min and the supernatant was collected and measured for substance P by ELISA (R&D Systems Europe Ltd., Abingdon, UK).

### *Spontaneous weight-bearing test*

MIA-induced arthritis was triggered by injecting MIA (500 μg) diluted in 40 μl of sterile endotoxin free PBS into the randomized hind knee joint of an anesthetized mouse. The contralateral knee joint was injected with the corresponding volume of the vehicle. The willingness to bear weight on the affected joint (spontaneous weight-bearing test) was measured with an incapacitance meter (IITC Life Science, Woodland Hills, CA, USA) for up to 28 days and compared to the baseline value. The mice were habituated in the measurement room for 60 min prior to the measurement and the subsequent measurements were carried out at the same time of the day. In order to obtain reliable data on the weight distribution, each mouse was measured eight times for 1 s at each time point with the measurer blinded to the affected limb.

### *Histological analysis*

MIA-induced OA was induced as described above. On day 28, the mice were sacrificed and the MIA and vehicle injected knee joints were dissected and fixed for 24 h in 10% formaldehyde, decalcified in Osteomoll (Merck, Darmstadt, Germany) for 48 h and embedded in paraffin. Coronal sections (5 μm thick) of femoro-tibial joints were rehydrated in a graded series of ethanol and stained with Safranin-O-Fast-Green. The cartilage changes were scored according to the OARSI guidelines[21] by two independent observers who were blinded to the treatment and genotype.

*Human chondrocyte culture and reverse transcription polymerase chain reaction (RT-PCR) measurements*

Leftover pieces of OA cartilage from knee joint replacement surgery were used under full patient consent and approval by the Ethics Committee of Tampere University Hospital, Tampere, Finland. Chondrocytes were isolated and cultured as described in the Supplementary data. The chondrocytes were incubated with MIA (100 μM), TRPA1 antagonist HC-030031 (100 μM), IL-1β (100 pg/ml, R&D Systems Europe Ltd.) or with combinations of these compounds for 6 h and analyzed by RT-PCR for the expression of COX-2 as described in the Supplementary data.

*Mouse cartilage culture and Western Blotting measurements*

Full-thickness articular cartilage from the femoral heads were cultured as described in the Supplementary data. The cartilage pieces were exposed to MIA (10 μM), TRPA1 antagonist HC-030031 (100 μM), IL-1β (100 pg/ml, R&D Systems Europe Ltd.) or to combinations of these agents for 24 h and the samples were analyzed for COX-2 by Western Blotting as described in the Supplementary data.

*$Ca^{2+}$-influx measurements*

TRPA1 mediated $Ca^{2+}$-influx was measured in HEK 293 cells[22] transiently transfected with human TRPA1 as described previously[11]. Briefly, cultured cells were loaded with fluo-3-acetoxymethyl ester (4 μM) and 0.08% Pluronic F-127® in Hanks' balanced salt solution (HBSS) containing 1 mg/ml of bovine serum albumin, probenecid (2.5 mM) and HEPES (25 mM, pH 7.2) for 30 min at room temperature. The intracellular free $Ca^{2+}$ levels were assessed with Victor3 1420 multilabel counter (Perkin Elmer, Waltham, MA, USA) at excitation/emission wavelengths of 485/535 nm[23]. In the experiments, the cells were first pre-incubated with the TRPA1 antagonist HC-030031 (100 μM) or the vehicle for 30 min at +37°C. Subsequently, MIA (100 μM) or AITC (50 μM) was added and the measurements were continued for 30 s after which a robust $Ca^{2+}$-influx was induced by application of the control ionophore compound, ionomycin (1 μM).

*Statistical analysis*

Results are expressed as mean ± 95% confidence interval. Data were analysed with SPSS 21 software (SPSS Inc, Chicago, IL, USA) with the tests used being detailed in the figure legends.

## Results

*MIA induces an acute inflammatory response in a TRPA1 dependent manner*

Injection of MIA into the mouse paw induced an acute inflammatory edema as can be seen in Fig. 1(A)–(C). Interestingly, treatment with the selective TRPA1 antagonist TCS 5861528 significantly decreased the formation of the edema (45% and 46% inhibition at 3 h and 6 h time points, respectively; Fig. 1(A)), suggesting that the response was mediated through activation of TRPA1. To prove the involvement of TRPA1 in the MIA-induced paw edema, we compared the responses between TRPA1 deficient and corresponding wild type mice. In confirmation of the results obtained with the TRPA1 antagonist, the MIA-induced inflammatory edema was significantly reduced in TRPA1 deficient mice as compared to wild type mice, showing a 58% lower response at 3 h and a 63% reduction at 6 h [Fig. 1(B)].

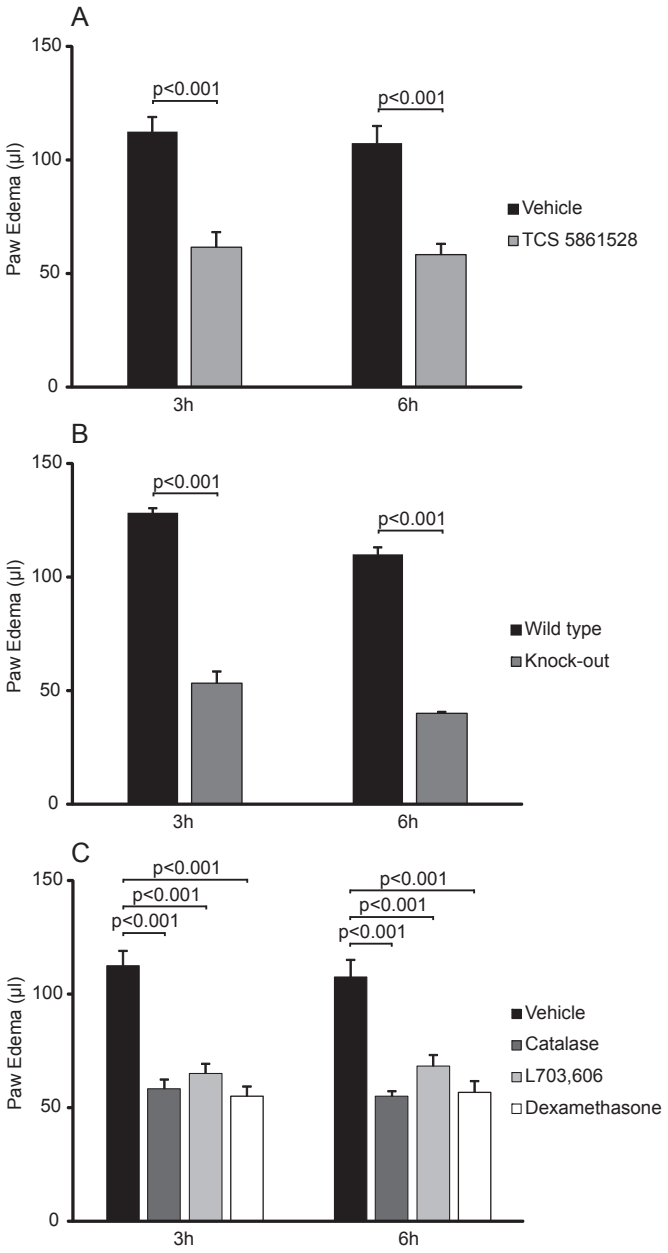


**Fig. 1.** MIA-induced acute paw edema was inhibited by both genetic depletion and pharmacological inhibition of TRPA1 as well as with treatment with the hydrogen peroxide detoxifying enzyme catalase and also the neurokinin 1 receptor antagonist. MIA induced an acute inflammatory edema when injected into the mouse hind paw. Treatment with the TRPA1 antagonist, TCS 5861528 (10 mg/kg) significantly inhibited the MIA-induced mouse paw edema (A). Similarly, TRPA1 deficient (knock-out) mice developed an alleviated edema in response to MIA as compared to the corresponding wild type mice (B). Furthermore, treatment with the hydrogen peroxide detoxifying enzyme, catalase (300 IU/paw) and the neurokinin 1 receptor antagonist, L703,606 (10 mg/kg) inhibited the development of MIA-induced edema (C) similar to treatment with the TRPA1 antagonist TCS 5861528. Administration of the glucocorticoid dexamethasone (2 mg/kg), used as an anti-inflammatory control compound, also inhibited MIA-induced inflammatory edema (C). The paw volume was measured with a plethysmometer before and 3 and 6 h after MIA injection (400 μg dissolved in 40 μl of endotoxin-free phosphate buffered saline). The contralateral control paw injected with the solvent of MIA (endotoxin-free phosphate buffered saline) developed no measurable edema. Paw edema is expressed as the volume change as compared to the pre-treatment value and the results are displayed as mean + 95% confidence interval, in A and C there were eight animals per group and in B there were six animals per group. Data were analysed with one-way ANOVA followed by Bonferroni's multiple comparison test.

The underlying mechanisms of MIA-induced acute inflammatory edema were first examined by treating the animals with the hydrogen peroxide degrading enzyme catalase. Based on the fact that MIA induces production of ROS[7] which also are known to activate TRPA1[10,16], we hypothesized that the generation of hydrogen peroxide could mediate the inflammatory reaction induced by the injection of MIA. Interestingly, catalase caused a clear decrease in MIA-induced paw edema (48% and 49% inhibition at 3 h and 6 h time points, respectively) as seen in Fig. 1(C).

The inflammatory effects of TRPA1 activation are frequently explained by the release of neuropeptides, especially substance P, and the promotion of neurogenic inflammation[9,10]. Therefore, we investigated the effects of the blockade of the receptor for substance P by using the neurokinin 1 receptor antagonist L703,606[20]. As seen in Fig. 1(C), treatment with L703,606 inhibited the MIA-induced paw edema by 42% at 3 h and by 36% at 6 h time points. Furthermore, MIA induced an increase in substance P levels in the inflamed paw tissue as shown in Fig. 2(A)–(B) and this increase was attenuated in TRPA1 knock-out mice [Fig. 2(A)]. Accordingly, pretreatment with the TRPA1 antagonist TCS 5861528 reduced the MIA-induced increase in substance P levels whereas L703,606 had no effect, as expected [Fig. 2(B)].

*MIA induces COX-2 expression in chondrocytes in a TRPA1 dependent manner*

Activation of TRPA1 has been reported to enhance the expression of the inducible prostaglandin synthase, COX-2, in some cell types[11]. MIA has also been found to increase COX-2 expression in OA joints[24,25]. Therefore we decided to investigate whether the pattern described above (i.e., that TRPA1 mediates the MIA-induced acute inflammatory edema) could also be extended to MIA-induced responses in human chondrocytes, focusing on COX-2 expression.

We cultured primary chondrocytes derived from patients with OA and used RT-PCR to confirm that TRPA1 was expressed in these cells. Next, we treated the chondrocytes with MIA, the selective TRPA1 antagonist HC-030031 or their combination with and without interleukin-1β (IL-1β) stimulation. MIA or HC-030031 alone did not alter COX-2 expression. Nonetheless, MIA clearly increased COX-2 expression in IL-1β stimulated chondrocytes. Furthermore, treatment with the TRPA1 antagonist HC-030031 abolished the MIA-induced increase in COX-2 expression as shown in Fig. 3(A).

The finding described above suggests that MIA can induce COX-2 expression in OA chondrocytes in a TRPA1-mediated manner. The mediator role of TRPA1 in that process was elucidated by determining COX-2 expression in articular cartilage from wild type and TRPA1 deficient mice. In support of the findings obtained in primary human OA chondrocytes, MIA increased COX-2 expression in IL-1β-stimulated cartilage from wild type [Fig. 3(B)] but not from TRPA1 knock-out [Fig. 3(C)] mice, and furthermore in the wild type mice, the effect of MIA was inhibited by the TRPA1 antagonist HC-030031 [Fig. 3(B)].

*Attenuation of MIA-induced joint pain and cartilage changes in TRPA1 deficient mice*

As a consequence of the acute inflammation, the development of cartilage degradation and joint pain are characteristic features encountered in MIA-induced OA. The latter is usually measured by an incapacitance meter in the so-called weight-bearing test[3,6]. As TRPA1 has been reported to mediate various forms of neuropathic and inflammatory pain[9,10], we investigated whether TRPA1 was also involved in the development of the MIA-induced joint pain. When MIA was injected into one knee joint, wild type mice developed a reduction in spontaneous weight-bearing on the affected joint, indicative of joint pain. Interestingly, an attenuated response was detected in TRPA1 deficient mice as seen in Fig. 4(A)–(B), suggesting that TRPA1 is indeed involved in the development of the joint pain typical of MIA-induced OA. Furthermore, the score of MIA-induced histological changes in the cartilage according to the OARSI guidelines[21] was lower in TRPA1 deficient than in wild type mice (Fig. 5).

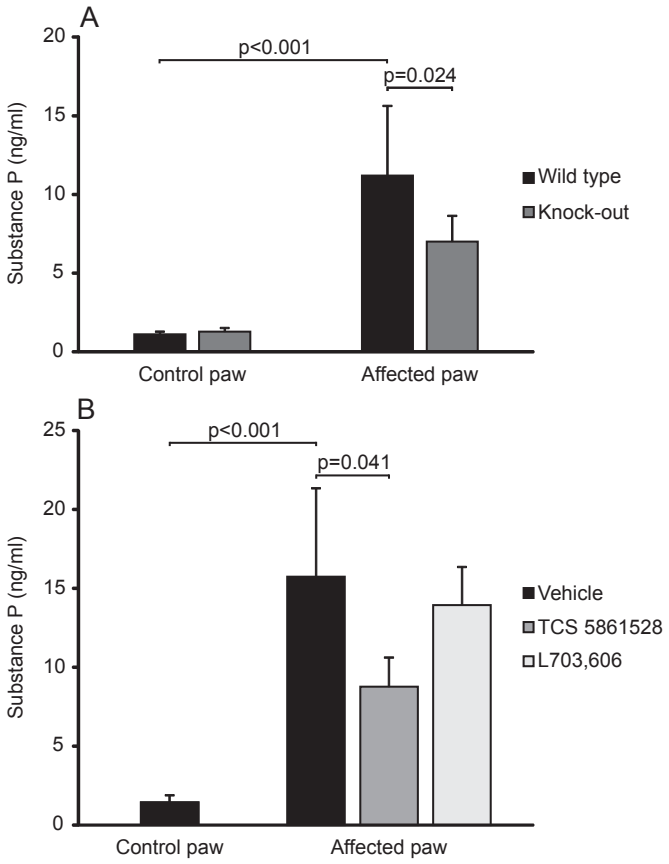


**Fig. 2.** MIA induced the release of the neuropeptide substance P into the inflamed paw; this effect was inhibited by both genetic depletion and pharmacological inhibition of TRPA1 but not by treatment with the neurokinin 1 receptor antagonist. Substance P concentrations were elevated in the mouse paw following an injection of MIA. Interestingly, substance P release was lower in TRPA1 deficient (knock-out) mice as compared to the corresponding wild type mice (A). Similarly, treatment with the TRPA1 antagonist TCS 5861528 (10 mg/kg) inhibited the release of substance P whereas the neurokinin 1 receptor antagonist L703,606 (10 mg/kg) had a negligible effect (B). The affected paw of the studied mice was injected with MIA (400 μg dissolved in 40 μl of endotoxin-free phosphate buffered saline) whereas the contralateral control paw was injected with the solvent (endotoxin-free phosphate buffered saline). After 6 h, the mice were sacrificed and the collected paw tissue samples were analysed for substance P concentrations by ELISA. The results are displayed as mean + 95% confidence interval, in (A) there were six animals per group and in (B) eight animals per group. Data were analysed with one-way ANOVA followed by Bonferroni's multiple comparison test.

*MIA is not a direct activator of TRPA1 ion channels*

As many of the MIA-induced responses were found to be mediated through TRPA1, we asked the question if MIA could be a direct activator of TRPA1 ion channels. To investigate that possibility, MIA was introduced to HEK 293 cells transfected with TRPA1. MIA did not induce $Ca^{2+}$-influx into the studied cells whereas the known TRPA1 agonist AITC induced an intense $Ca^{2+}$-influx which

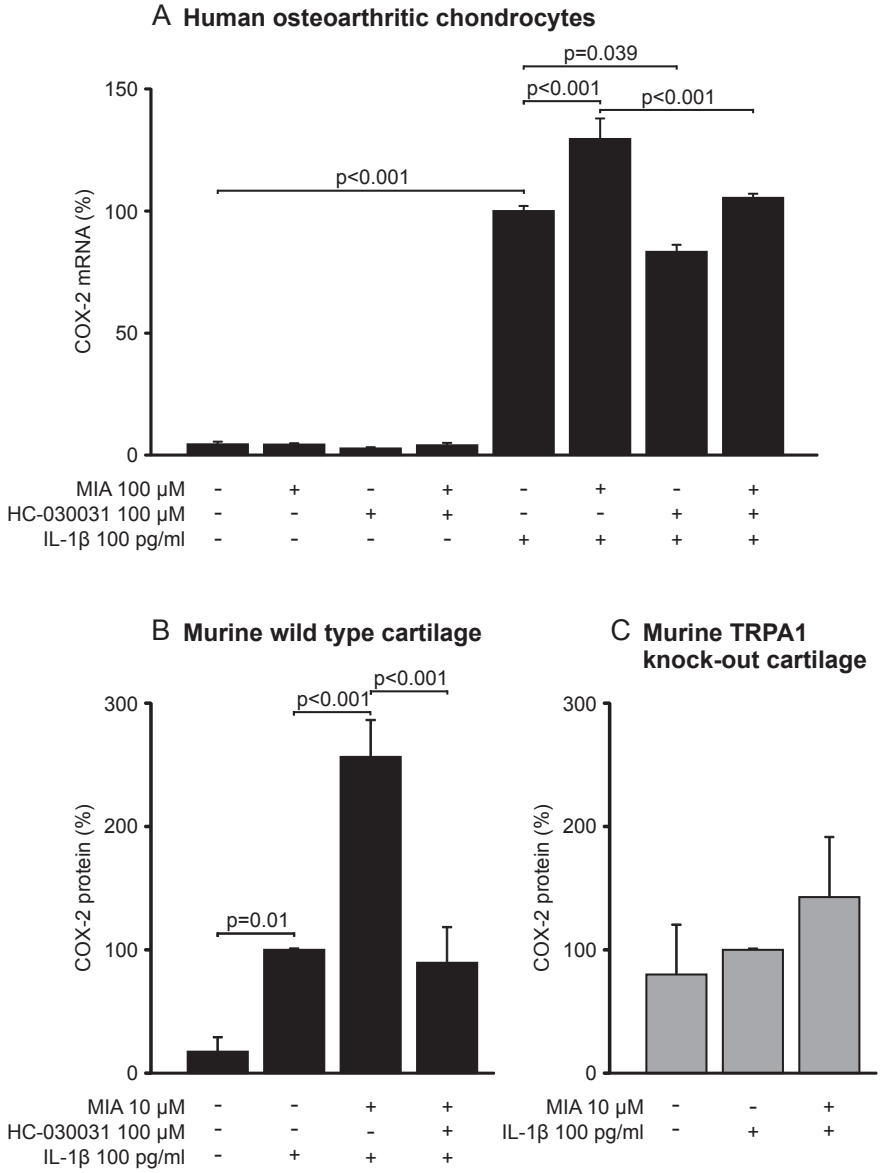


**Fig. 3.** MIA-induced COX-2 expression was reduced by pharmacological inhibition and genetic depletion of TRPA1. In primary cultures of human osteoarthritic chondrocytes, treatment with the TRPA1 antagonist HC-030031 reduced the expression of the proinflammatory gene COX-2 induced by co-stimulation with MIA and IL-1β (A). Correspondingly in the cartilage derived from mice, MIA increased COX-2 expression in IL-1β stimulated cartilage from wild type but not from TRPA1 deficient (knock-out) mice (B). The samples were cultured with the studied compounds for 6 h and analyzed for COX-2 mRNA expression by RT-PCR (A) or for 24 h and analyzed for COX-2 protein expression by Western Blotting (B). The mRNA expression was normalized against housekeeping gene GAPDH (A) and the protein expression against loading control actin (B). The response in IL-1β treated chondrocytes/cartilage was set at 100% and the other results were calculated in relation to that value. The results are displayed as mean + 95% confidence interval. The human samples were obtained from six different donors and the experiments were performed in triplicate (A). The cartilage samples were from five mouse ($n = 5$) in each treatment (B and C). Data were analysed with SPSS software with one-way ANOVA followed by Bonferroni's multiple comparison test.

could be inhibited by treatment with the TRPA1 antagonist HC-030031 as seen in Fig. 6. These data indicate that MIA is not a direct TRPA1 agonist but most likely induces a release of endogenous TRPA1 activators which are responsible for the TRPA1-mediated effects of MIA discovered in the present study.

## Discussion

The present study revealed that MIA-induced acute inflammation was reduced in TRPA1 deficient mice as well as by the treatment with the TRPA1 antagonist TCS 5861528, the neurokinin 1 receptor antagonist L703,606 and the $H_2O_2$ degrading enzyme catalase. We also demonstrated that the spontaneous weight shift away from the MIA-injected limb was attenuated in TRPA1 deficient mice when compared to the corresponding wild type mice. Furthermore, MIA-induced cartilage changes were less severe in TRPA1 deficient mice. These results together suggest that the TRPA1 ion channel is significantly involved in the development of MIA-induced acute inflammation, cartilage changes and joint pain.

Originally TRPA1 was described in fetal lung fibroblasts in 1999[26]. Thereafter it has been shown that TRPA1 is expressed in different afferent sensory neurons such as Aδ- and C-fibers[27]. Moreover, substantial non-neuronal expression and function of TRPA1 have been identified in lining cells such as keratinocytes, synoviocytes and endothelial cells[10,28]. The physiological role of TRPA1 is believed to be in the sensing of exogenous irritating and

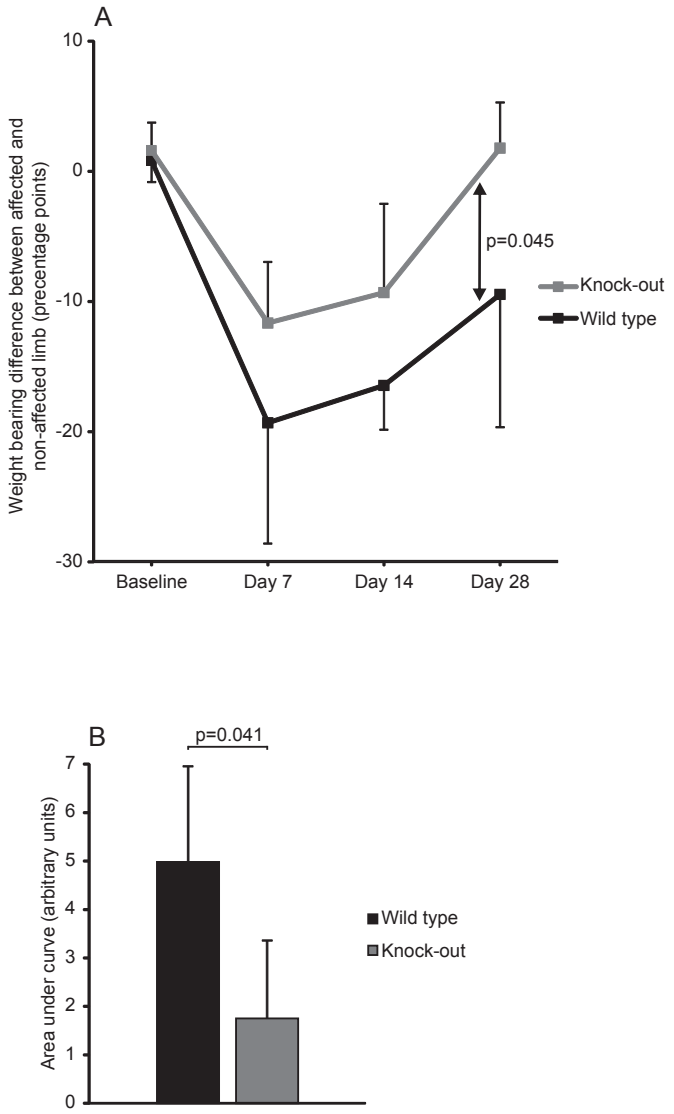


**Fig. 4.** TRPA1 deficient mice exhibited an attenuated response to MIA in the weight-bearing test indicative of alleviated joint pain. When MIA was injected into one knee joint, wild type mice exhibited a reduction in spontaneous weight-bearing on the affected limb indicative of joint pain. Interestingly, an attenuated weight distribution change was seen in TRPA1 deficient (knock-out) mice. Spontaneous distribution of weight between the hind limbs was measured with an incapacitance meter before and at 7, 14 and 28 days after intra-articular injection of MIA (500 μg dissolved in 40 μl of endotoxin free phosphate buffered saline). The contralateral knee joint was injected with the solvent of MIA (endotoxin-free phosphate buffered saline) and the measurer was blinded for the affected limb. In (A), results are displayed as difference of percentage points between the weight bore by the affected and non-affected limb and given as mean + 95% confidence interval, $n = 7$ animals in wild type and TRPA1 deficient groups. Data were analysed by using mixed between-within subjects ANOVA and a statistically significant difference between the two genotypes was found ($P = 0.045$). In (B), the area-under-the curve (AUC) for the shift from the equal balance between the hind limbs was calculated, and the results of wild type and TRPA1 deficient mice are presented as mean + 95% confidence interval, $n = 7$ animals in wild type and TRPA1 deficient groups. Data were analysed by using unpaired *T*-test and a statistically significant difference between the two genotypes was found ($P = 0.041$).

noxious compounds, but there is evidence accumulating that TRPA1 possesses also the ability to promote inflammation[9,10]. TRPA1 has been proven to be crucial for the development of inflammatory edema[11], hyperalgesia[12] and pain[13,14] in pathological conditions such as airway hyperreactivity and inflammation[29,30], acute gouty arthritis[31] and neurogenic inflammation associated with colitis[32]. In addition to the fact that TRPA1 is activated by endogenously formed reactive oxygen and nitrogen species and their metabolites[16], TRPA1 is also sensitized by several secondary messengers, i.e., phospholipase C and protein kinase A[33], which are activated by various proinflammatory mediators through their G-protein coupled receptors. Activation of TRPA1 primarily leads to pain sensation and amplification of neurogenic inflammation by promoting the release of neuropeptides such as substance P and calcitonin-gene related peptide[9,10]. In addition, many other secondary inflammatory mechanisms following TRPA1 activation have been identified. For example, inflammatory edema caused by TRPA1 activation is mediated through mechanisms such as mast cell degranulation, neutrophil migration, the release of histamine, serotonin and adrenaline as well as the production of prostaglandins[11,34]. In addition, the activation of TRPA1 has been shown to enhance the expression of inflammatory genes such as prostaglandin producing enzyme COX-2, myeloperoxidase and IL-1β[11,35–37].

An injection of MIA into a rodent's knee joint is a widely used experimental model to study OA as it triggers the changes resembling the histological and pathophysiological features of the human disease[38–41]. In addition, pain-like behavior appears within a few weeks; this is commonly evaluated by the weight-bearing test[38,39,41]. The mechanism of action of MIA has been attributed to inhibition of glyceraldehyde-3-phosphate dehydrogenase. This disrupts the glucose metabolism of chondrocytes, leading to ROS production and caspase activation, and further to the catabolism of cartilage matrix and cell death which can be detected both *in vivo* and *in vitro*[7,38,42,43]. In addition to evoking cartilage degradation, MIA induces an acute inflammation which is associated with edema formation and increased expression of proinflammatory factors such as IL-1β, IL-6, IL-15, inducible nitric oxide synthase (iNOS), COX-2 and metalloproteinase-13[24,25]. Interestingly, MIA has also been demonstrated to trigger an early release of neuropeptides, substance P and calcitonin gene related peptide[44].

Even though the role of TRPA1 in MIA-induced inflammation and cartilage changes has previously been unknown, there are two studies which have investigated TRPA1 in MIA-induced OA, although focusing on pain. Curiously, a single dose of TRPA1 antagonist given shortly prior to the measurements failed to alter pain-like behaviour or cause any change in weight-bearing test at the later stage of the arthritis[6] but it was effective in blocking mechanically induced hypersensitivity[19]. However, these studies focused mainly on noxious neuronal signals and pain mediated by activation of TRPA1, but they did not examine acute inflammation or the long-term effect of TRPA1 on the development of the joint pain or cartilage changes induced by MIA. In the current study, we were able to link the activation of TRPA1 to the formation of MIA-induced acute inflammation and cartilage changes and we also observed a diminished weight shift in TRPA1 knock-out mice reflecting an alleviation of joint pain.

The present results demonstrate that the MIA-induced acute inflammatory paw edema was dependent on TRPA1 activation. Both genetic depletion and pharmacological inhibition of TRPA1 were highly effective in reducing the edema. Furthermore, the extent of the edema was also reduced by treatment with the hydrogen peroxide detoxifying enzyme catalase indicating that the edema was likely attributable to hydrogen peroxide. The edema formation was also reversed by treatment with L703,606, a compound which antagonises the neurokinin 1 receptor known to be the main receptor for substance P. Importantly, when the concentrations of substance P were assayed in the inflamed paw tissue, they were found to be diminished in the TRPA1 knock-out mice as compared to the wild type mice as well as in mice treated with the TRPA1 antagonist TCS 5861528, but as expected, treatment with

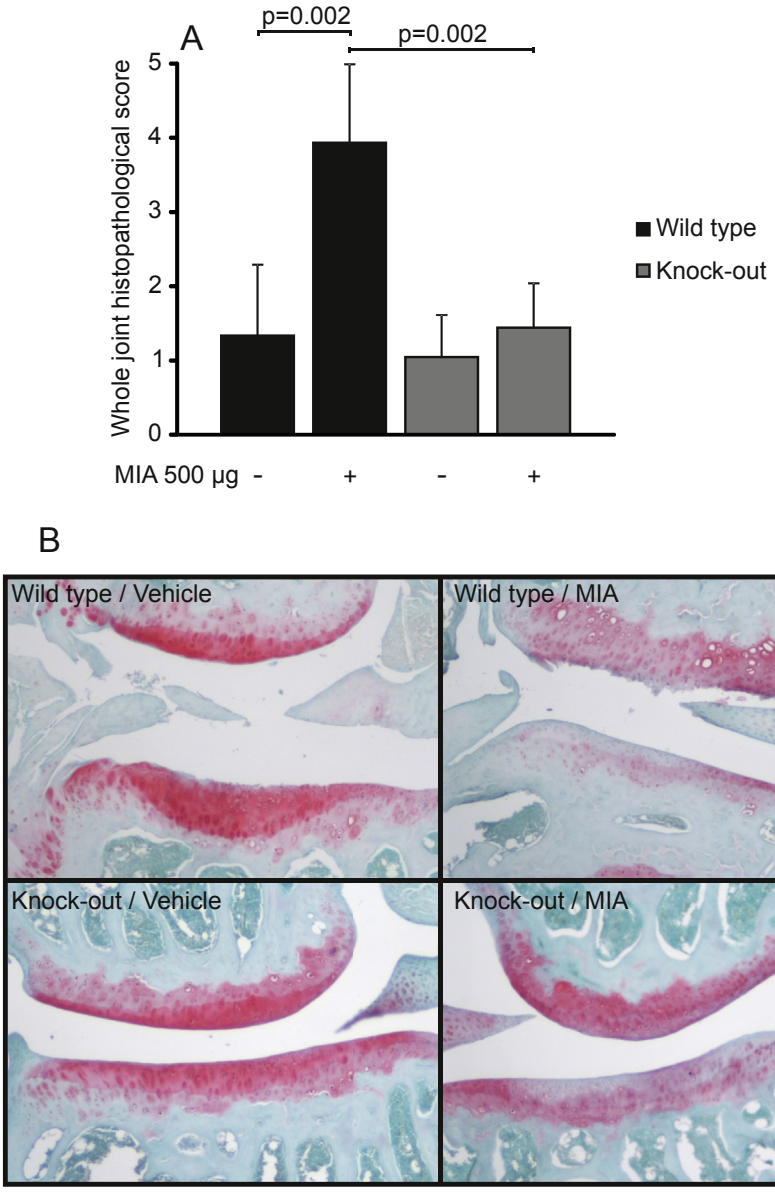


**Fig. 5.** TRPA1 deficient mice developed less severe cartilage changes in the knee joint following MIA injection. When MIA was injected into the knee joint, wild type mice developed more severe cartilage changes than TRPA1 deficient (knock-out) mice. Twenty-eight days after intra-articular injection of MIA (500 µg dissolved in 40 µl of endotoxin-free phosphate buffered saline) in one knee joint and the solvent of MIA (endotoxin-free phosphate buffered saline) in the contralateral joint, mice were sacrificed and the knee joints were dissected for histology. The cartilage changes were scored according to the OARSI guidelines. In (A), the whole joint histopathological score is presented. The results are displayed as mean + 95% confidence interval, $n = 7$ animals in wild type and TRPA1 deficient groups. Data were analysed with SPSS software by using one-way ANOVA with Bonferroni's multiple comparison test. In (B), representative figures of MIA-injected and vehicle-injected (control) contralateral knee joints from wild type and TRPA1 deficient (knock-out) mice are shown. The sections were stained with Safranin-O-Fast-Green, magnification × 10.

L703,606 did not alter the levels of substance P. Based on the present results, the likely sequence of events is that first MIA induces the formation of ROS in the target cells; second, the ROS activate TRPA1 leading to release of substance P; and third, the released substance P activates the neurokinin 1 receptor to induce the acute inflammatory response. Within a few weeks after the intra-articular injection of MIA, one can see evidence of degenerating cartilage and joint pain and both of those responses appear to be alleviated in TRPA1 deficient mice. Based on the present data, it is tempting to propose causality between the MIA-induced acute inflammation and the subsequent OA-like changes, but further studies will be needed to reveal their association.

Even though classically the function of TRPA1 has mainly been studied in sensory nerves, non-neuronal expression and function of TRPA1 are now clearly recognized[28]. In the present study, we observed the expression of TRPA1 in human primary chondrocytes derived from OA patients. In view of the known effects of MIA on chondrocytes[25], we decided to study the possible effects of TRPA1 in cultured human chondrocytes. When the cells were stimulated with MIA and IL-1β, the expression of the proinflammatory gene COX-2 was increased and this could be inhibited by treatment with the TRPA1 antagonist HC-030031. The results were in parallel with findings in cultured murine cartilage; MIA induced a clear increase in COX-2 expression in IL-1β-stimulated cartilage from wild type mice but this effect was not seen in cartilage from TRPA1 deficient mice. These results were comparable to those obtained earlier in TRPA1 transfected HEK 293 cells[11] indicating that the proinflammatory properties of TRPA1 might not be exclusively due to its

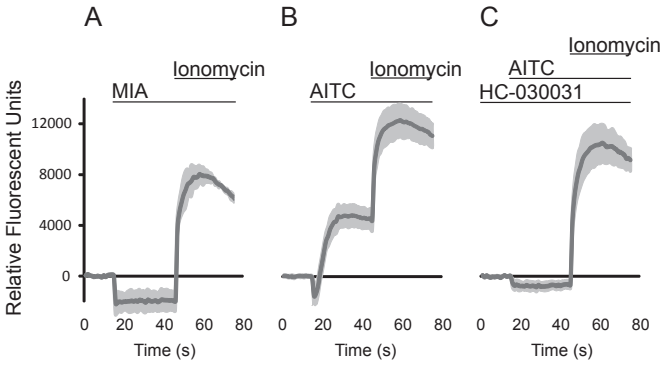


**Fig. 6.** MIA did not induce TRPA1 mediated $Ca^{2+}$-influx into HEK 293 cells transfected with TRPA1. MIA did not evoke TRPA1-mediated $Ca^{2+}$-influx (A) whereas the known TRPA1 agonist AITC induced a robust $Ca^{2+}$-influx (B) which was inhibited by pre-treatment with the selective TRPA1 antagonist HC-030031 (C). HEK 293 cells were transfected with plasmids encoding TRPA1 and loaded with Fluo-3 AM as described in the Methods. The intracellular $Ca^{2+}$ concentration was measured by Victor3 multilabel counter at excitation/emission wavelengths of 485/535 nm at 1/s frequency. The cells were first pre-incubated with HC-030031 (100 μM) (C) or the vehicle (A–B) for 30 min at +37°C. In the experiments (A–C), basal fluorescence was first measured for 15 s and thereafter 100 μM of MIA (A) or 50 μM of AITC (B–C) was added and the measurement was continued for 30 s after which 1 μM of the control ionophore compound, ionomycin, was introduced to the cells. The results were normalized against background and expressed as mean (dark gray line) ± 95% confidence interval (light gray shadowing), $n = 6$.

neuronal functions. One can only speculate on the mechanisms explaining how TRPA1 activation results in increased COX-2 expression but intracellular $Ca^{2+}$ levels may be the link. TRPA1 activation elevates the intracellular $Ca^{2+}$ concentration which may have either a direct or an indirect effect on the expression of inflammatory genes including COX-2 as reported previously[45–47]. It is noteworthy however, that substance P is expressed in chondrocytes and this neuropeptide modulates their functions[48] and therefore the effects seen could also be due to autocrine signalling.

When we scaled up the study setting and analysed the MIA-induced joint pain with the weight-bearing test, we found that the pain response was attenuated in TRPA1 deficient mice. The MIA-induced weight-bearing test is often performed in rats since these animals display a larger change in weight distribution between their lower limbs than mice and this should be considered when interpreting the present results. Despite this limitation, the use of mice was justified in the current study because the mouse is the only available TRPA1 deficient species. The weight distribution change reported here in wild type mice was of a similar magnitude as reported previously with this model[49]. Although the pathogenesis of the MIA-induced joint pain is not fully understood, it could be initiated and then modified by the early inflammation induced by MIA[3,49] and the present results clearly show a diminished acute inflammation in response to MIA in TRPA1 deficient mice. Arthritis involves many cell types including neurons, chondrocytes and synoviocytes all of which express TRPA1 and its activation may trigger or regulate the pathogenesis of arthritis. Interestingly, neuropeptides are known to be involved in arthritis[50] and for example the expression of the receptor for substance P, i.e., the neurokinin 1 receptor, is abundant[48]. Therefore it is clearly possible that activation of TRPA1 resulting in the release of neuropeptides such as substance P plays a role in the pathogenesis of OA.

At the moment, the therapy of OA is based on analgesic drugs, physical exercise, reduction of overweight and ultimately joint replacement surgery. Unfortunately, no effective disease modifying drugs are available. The present study introduces TRPA1 as a factor involved in mediating the acute inflammation and development of cartilage changes and joint pain in MIA-induced experimental OA. As the experimental model mimics many of the features of human OA, the present results raise the possibility that TRPA1 may play a central role also in the pathogenesis of OA and thus provide a novel target for analgesic and anti-inflammatory drugs with disease modifying potential.

## Author contributions

LJM contributed to the conception and design of the study, to the acquisition, analysis and interpretation of data and drafted the manuscript. MH contributed to the conception and design of the study and to the acquisition, analysis and interpretation of data. EN, PI and KV contributed to the acquisition, analysis and interpretation of data. RMN contributed to the conception and design of the study and to the acquisition, analysis and interpretation of data. LL contributed to the conception and design of the study and to the analysis and interpretation of data. EM supervised the study and contributed to the conception and design of the study and in the analysis and interpretation of data. All authors contributed to revising the manuscript critically for important intellectual content and have approved the final version of the manuscript for submission. The first author LJM (lauri.j.moilanen@uta.fi) and the corresponding author EM (eeva.moilanen@uta.fi) take responsibility for the integrity of the work as a whole, from inception to finished article.

## Conflict of interest statement

The authors declare no conflicts of interests.

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## Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.joca.2015.09.008.

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