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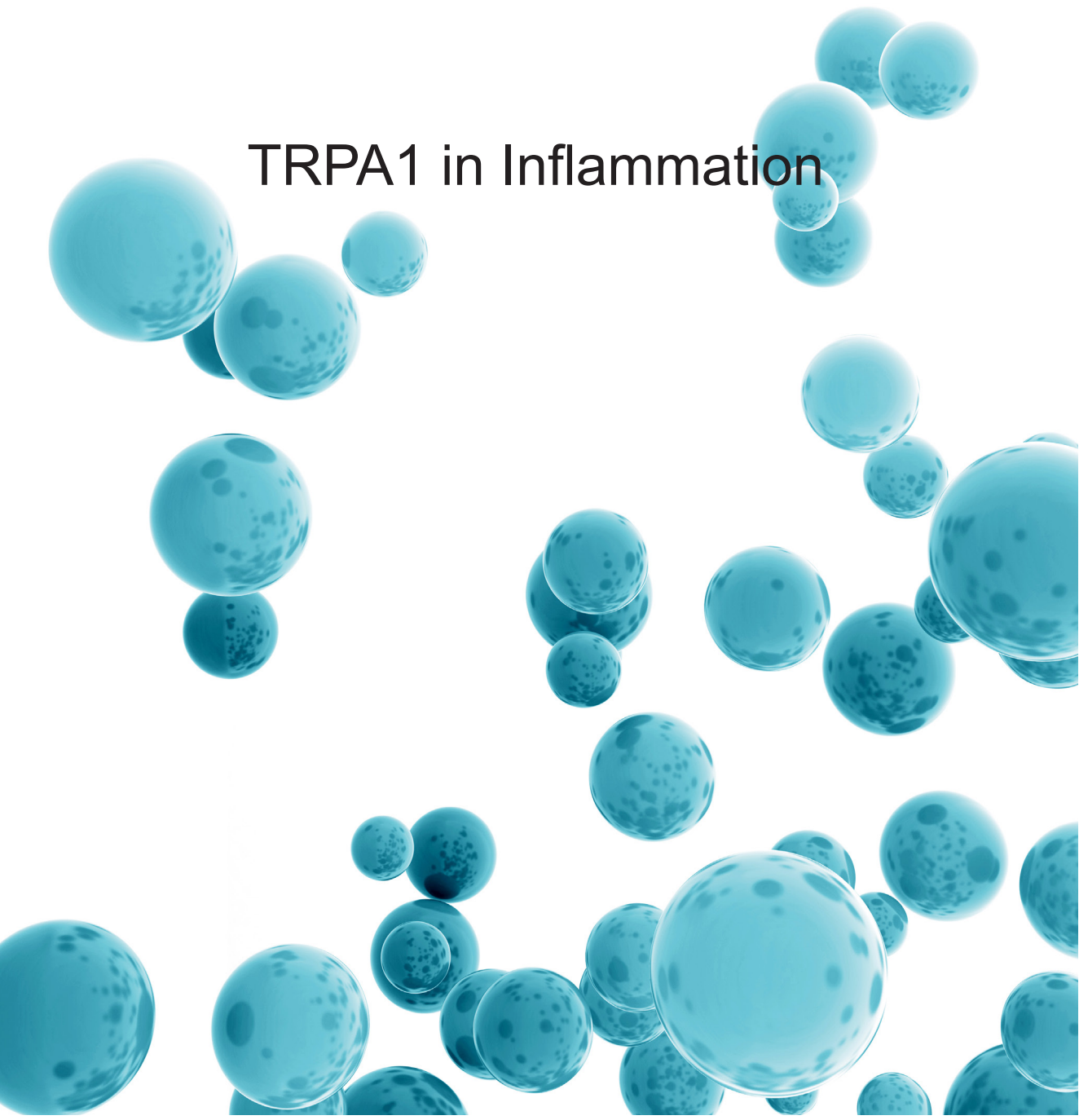
LAURI MOILANEN

TRPA1 in Inflammation



LAURI MOILANEN

TRPA1 in Inflammation

ACADEMIC DISSERTATION
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UNIVERSITY OF TAMPERE

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University of Tampere, School of Medicine
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Finland

Supervised by
Docent Riina Nieminen
University of Tampere
Finland
Professor Lauri Lehtimäki
University of Tampere
Finland
Professor Eeva Moilanen
University of Tampere
Finland

Reviewed by
Docent Paula Rytilä
University of Helsinki
Finland
Professor Ulf Simonsen
Aarhus University
Denmark

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To my close ones.

Contents

List of Original Communications

This thesis is based on the following original communications:

I Moilanen LJ, Laavola M, Kukkonen M, Korhonen R, Leppänen T, Högestätt ED, Zygmunt PM, Nieminen RM, Moilanen E. TRPA1 Contributes to the Acute Inflammatory Response and Mediates Carrageenan-Induced Paw Edema in the Mouse. Sci Rep, 2012; 2: 380. Epub 2012 Apr 24.

II Moilanen LJ, Hämäläinen M, Lehtimäki L, Nieminen RM, Moilanen E. Urate Crystal Induced Inflammation and Joint Pain Are Reduced in Transient Receptor Potential Ankyrin 1 Deficient Mice – Potential Role for Transient Receptor Potential Ankyrin 1 in Gout. PLoS One, 2015; 10(2): e0117770. Epub 2015 Feb 6.

III Moilanen LJ, Hämäläinen M, Nummenmaa E, Ilmarinen P, Vuolteenaho K, Nieminen RM, Lehtimäki L, Moilanen E. Monosodium Iodoacetate-Induced Inflammation and Joint Pain Are Reduced in TRPA1 Deficient Mice - Potential Role of TRPA1 in Osteoarthritis. Osteoarthritis Cartilage, 2015; 23(11): 2017-2026.

IV Moilanen LJ, Hämäläinen M, Ilmarinen P, Kankaanranta H, Nieminen RM, Moilanen E, Lehtimäki L. Transient Receptor Potential Ankyrin 1 Enhances Ovalbumin-Induced Acute Allergic Inflammation in Murine Models. Submitted for publication.

V Moilanen LJ, Hämäläinen M, Lehtimäki L, Nieminen RM, Muraki K, Moilanen E. Pinosylvin Inhibits TRPA1-Induced Calcium Influx in Vitro and TRPA1-Mediated Acute Paw Inflammation in Vivo. Basic Clin Pharmacol Toxicol, 2016; 118(3): 238-242. Epub 2015 Oct 7.

Abbreviations

AM	Acetoxymethyl ester
AITC	Allyl isothiocyanate
CGRP	Calcitonin gene-related peptide
COX	Cyclooxygenase
DMEM	Dulbecco's Modified Eagle's Medium
ELISA	Enzyme-linked immunosorbent assay
EMEM	Eagle's Minimum Essential Medium
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
HC-030031	Selective TRPA1 blocker; 2-(1,3-Dimethyl-2,6-dioxo-1,2,3,6-tetrahydro-7H-purin-7-yl)-N-(4-isopropylphenyl)acetamide
IgE	Immunoglobulin E
IL	Interleukin
iNOS	Inducible nitric oxide synthase
KO	Knock-out
L703,606	NK1 receptor blocker; cis-2-(Diphenylmethyl)-N-[(2-iodophenyl)methyl]-1-azabicyclo[2.2.2]octan-3-amine oxalate salt
MCP-1	Monocyte chemotactic protein-1
MIA	Monosodium iodoacetate
MIP-1α	Macrophage inflammatory protein-1α
MIP-2	Macrophage inflammatory protein-2
MPO	Myeloperoxidase
MSU	Monosodium urate
NF-κB	Nuclear factor-κB
NK1 receptor	Neurokinin-1 receptor
NO	Nitric oxide
PBS	Phosphate buffered saline
PIP_2	Phosphatidylinositol-4,5-bisphosphate
PKA	Protein kinase A
PLC	Phospholipase C
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute culture medium
RT-PCR	Reverse transcription polymerase chain reaction
SEM	Standard error of the mean
TCS 5861528	Selective TRPA1 blocker; 2-(1,3-Dimethyl-2,6-dioxo-1,2,3,6-tetrahydro-7H-purin-7-yl)-N-[4-(1-methylpropyl)phenyl]acetamide
T_H2	T helper 2 cell
TNF-α	Tumor necrosis factor-α
TRP	Transient receptor potential
TRPA1	Transient receptor potential ankyrin 1
TRPV1	Transient receptor potential vanilloid 1
WT	Wild type

Abstract

Inflammation is part of the host defence mechanisms meant to protect the host from invading pathogens such as bacteria, viruses and parasites and to restore tissue and system homeostasis. Inflammation evokes symptoms like local pain, edema, and redness especially in the acute phase, but the reaction may continue and turn into a chronic state, capable of leading to irreversible changes followed by a loss-of-function. Sometimes, the regulation and targeting of inflammation is inappropriate and may prove harmful to the host. Inflammatory diseases are responsible for much human suffering and pose a major economic burden. Therefore, better treatment methods for inflammation are sought continuously.

Transient receptor potential ankyrin 1 (TRPA1) is a member of the large family of TRP ion channels. The ligand gated and membrane bound TRPA1 is permeable to ions, predominantly to Ca^{2+}. Initially TRPA1 was found to mediate nociception in sensory nerves and serve as a chemosensor for potentially harmful exogenous compounds, but later the understanding has expanded. In addition to mediating pain, the activation of TRPA1 has been associated with inflammatory hyperalgesia, allodynia and extravasation. Unexpected ligands for TRPA1 have been discovered, as many inflammation associated endogenous factors such as reactive oxygen and nitrogen species (ROS and RNS) have been proven to activate TRPA1. Therefore, the role of TRPA1 might not be limited to chemosensation of exogenous irritants. The present studies utilized four experimental inflammatory disease models to clarify the potential proinflammatory role of TRPA1 activation.

The first study (I) revealed the crucial role of TRPA1 in the carrageenan-induced acute murine paw edema model, which is a general model of robust acute inflammation used widely in screening for anti-inflammatory drugs. Pharmacological inhibition and genetic depletion of TRPA1 attenuated the development of carrageenan-induced paw edema. Furthermore, TRPA1-mediated edema was linked to prostaglandin production, as the cyclooxygenase (COX) inhibitor ibuprofen was able to block the inflammatory edema induced by direct TRPA1 activation.

Gout flare is a condition associated with severe joint pain and acute inflammation and it was hypothetized that TRPA1 could be involved in this reaction. The second

study (II) investigated whether experimental murine gout would be enhanced by TRPA1 activation. Interestingly, it was found in several *in vivo* tests i.e. acute paw edema model, experimental gouty knee arthritis and the air-pouch inflammation test that inflammatory edema, arthritic pain and the production of proinflammatory cytokines in response to an injection of monosodium urate crystals were diminished by pharmacological inhibition and genetic depletion of TRPA1.

The putative relationship between osteoarthritis and TRPA1 was examined in the third study (III) based on the findings obtained with the monosodium iodoacetate (MIA) induced arthritis, a widely used experimental model of osteoarthritis. This model begins with an acute inflammatory phase which proceeds to degradation of articular cartilage and joint pain. The inhibition of TRPA1 both *in vivo* and *in vitro* exerted an anti-inflammatory effect in the acute setting and the longer evaluation of the test animals revealed alleviated joint pain and less severe cartilage degradation in TRPA1 deficient mice.

The fourth study (IV) evaluated the role of TRPA1 in acute allergic inflammation. Treating the studied mice with a TRPA1 blocker alleviated the acute allergic response induced by a specific allergen and the phenomenon duplicated in TRPA1 deficient mice. The effect was seen early in the acute allergic inflammation as less tissue edema and reduced production of the proinflammatory cytokine IL-4 and further as a decreased recruitment of eosinophils to the site of the inflammation. Importantly, genetic depletion of TRPA1 did not impair the splenocytes' response to the allergen proposing an unaltered sensitization.

As TRPA1 seemed a promising target to inhibit to alleviate acute inflammation and inflammatory pain, the fifth study (V) aimed at identifying a novel TRPA1 blocker. The stilbenoid pinosylvin was found to be a potent blocker of TRPA1-induced responses both *in vitro* and *in vivo* and therefore could serve as a basis for the development of new TRPA1 inhibiting drugs.

The present studies have produced valuable novel information on the proinflammatory role of TRPA1. The findings expand our understanding of the TRPA1 ion channel; it is no longer simply a nociceptor and chemosensor for potentially harmful exogenous compounds, instead it is a significant endogenous mechanism mediating and regulating inflammation and inflammatory pain in several disease states. The findings propose novel properties of TRPA1 in human pathophysiology and open new avenues for the treatment of inflammation and inflammatory pain through inhibition of TRPA1.

Tiivistelmä

Tulehdus on elimistön suojamekanismi ulkoisia taudinaiheuttajia vastaan. Sen tarkoituksena on poistaa uhkaava tekijä sekä palauttaa kohde-elimen ja elimistön tasapaino. Tulehdusreaktion oireita ovat punoitus, kuumotus, turvotus ja kipu. Immuunijärjestelmä pyrkii kohdentamaan tulehdusreaktion oikeisiin kohteisiin sopivissa mittasuhteissa, mutta häiriintyessään tulehdusreaktio voi olla elimistölle haitallinen ja johtaa tulehdustautien syntyyn. Globaalisti tulehdukselliset sairaudet aiheuttavat nykyhoidoista huolimatta paljon inhimillistä kärsimystä ja terveydenhuollon kustannuksia ja siksi niiden tutkimus parempien hoitojen kehittämiseksi on oleellisen tärkeää.

Transient receptor potential ankyriini 1 (TRPA1) kuuluu TRP-ionikanava-perheeseen yhdessä yli 30 muun proteiinin kanssa. TRPA1 sijaitsee solukalvolla ja aktivoituessaan päästää soluun pääasiassa Ca^{2+}-ioneja. TRPA1 ilmenee hermosolujen aistinpäätteissä ja sen fysiologinen tehtävä on aistia elimistölle haitallisia kemiallisia ärsykkeitä ja varoittaa niistä tuottamalla kipua. TRPA1:n aktivaatio välittää myös tulehdukseen liittyvää turvotusta sekä herkistymistä kivulle. Uudet tutkimustulokset osoittavat, että ärsyttävien kemikaalien lisäksi TRPA1 aktivoituu myös joidenkin tulehduksessa syntyvien molekyylien, kuten happi- ja typpiradikaalien, vaikutuksesta. Toisaalta on todettu, että myös eräät muut solutyypit kuin hermosolut ilmentävät TRPA1:tä. Siten on teoriassa mahdollista, että TRPA1:n aktivaatio olisi yleinen tulehdusta voimistava mekanismi. Tämän väitöskirjan osatöissä tutkimme TRPA1:n aktivaation roolia kokeellisesti neljän eri tulehdustaudin mallissa.

Ensimmäisessä osatyössä (I) tutkimme TRPA1:n aktivaation osuutta karrageeni-tulehduksessa. Tämä eläinkoe on akuutin tulehduksen yleinen koemalli mm. seulottaessa uusia anti-inflammatorisia lääkkeitä. Tulostemme mukaan karrageenin laukaisema akuutti tulehduksellinen tassuturvotus oli lievempi TRPA1:n estäjällä hoidetuilla sekä TRPA1-poistogeenisillä hiirillä. TRPA1:n vaikutus oli kytkeytynyt prostaglandiinituotantoon, sillä syklo-oksygenaasientsyymin estäminen vaimensi merkittävästi myös suoran TRPA1:n aktivaattorin laukaisemaa turvotusvastetta.

Toisessa osatyössä (II) tutkittiin TRPA1:n merkitystä kolmessa kihtiä kuvaavassa eläinmallissa. Uraattikiteiden laukaisema turvotus, nivelkipu ja tulehdussytokiinien

tuotto väheni merkittävästi TRPA1:n aktivaation estävällä lääkehoidolla sekä oli alentunut TRPA1-poistogeenisissä hiirissä.

Nivelrikon ja TRPA1:n välistä yhteyttä tutkittiin kolmannessa osatyössä (III). Natriumjodoasetaatin (engl. monosodium iodoacetate, MIA) laukaisema nivelrikon koe-eläinmalli on laajasti käytetty tutkimusmenetelmä, jossa MIA aiheuttaa ensin akuutin tulehduksen ja tämä johtaa nivelruston vaurioon ja kipuun. Tuloksemme osoittavat, että MIA:n laukaisema tulehdus on ainakin osittain TRPA1-välitteinen sekä *in vitro* että *in vivo* ja että nivelrikkomuutosten sekä nivelkivun kehittyminen on vähentynyt TRPA1-poistogeenisissä hiirissä.

Neljännessä osatyössä (IV) tutkimme TRPA1:n osuutta akuutissa allergisessa tulehduksessa. Tutkimusasetelmassa koe-eläimet herkistettiin allergeenille ja varsinaisissa kokeissa tutkimme allergeenialtistuksen käynnistämää tulehdusvastetta. TRPA1:n estäminen lääkkeellä ja ionikanavan geneettinen vaimentaminen lievittivät selvästi allergista tulehdusta: havaitsimme, että alkuvaiheen paikallinen turvotusvaste ja tulehdussytokiini IL-4:n tuotto vähenivät. Vuorokauden kuluttua tulehdusreaktio oli vaimeampi TRPA1:n estäjällä hoidetuilla sekä TRPA1-poistogeenisillä hiirillä, mistä osoituksena eosinofiilien kertyminen tulehdusalueelle oli vähentynyt. TRPA1-poistogeenisten hiirten pernan lymfosyyttien vaste allergeenille oli samanlainen kuin vastaavilla villityypin hiirillä. Tämä viittaa siihen, että TRPA1 ei vaikuta allergisoitumiseen vaan nimenomaan allergeenin aiheuttamaan tulehdusreaktioon.

Koska TRPA1:n aktivaation estäminen osoittautui tehokkaaksi keinoksi hoitaa akuuttia tulehdusta tutkimissamme tautimalleissa, pyrimme identifioimaan uusia TRPA1:n aktivaatiota estäviä molekyylejä. Aikaisempien seulontatutkimustemme perusteella selvitimme pinosylviinin tehoa TRPA1:n estäjänä (osatyö V). Pinosylviini esti TRPA1:n välittämiä vasteita sekä solu- että eläinkokeissa. Tulos antaa pohjaa tulevaisuuden lääkekehitykselle.

Tämän väitöskirjan tutkimustulokset tuovat uutta tärkeää tietoa TRPA1:n merkityksestä tulehdusreaktiota ja siihen liittyvää kipua voimistavana mekanismina, joka aktivoituu endogeenisten välittäjäaineiden vaikutuksesta. Nämä löydökset laajentavat merkittävästi vallitsevaa käsitystä TRPA1-ionikanavan merkityksestä ihmisen patofysiologiassa ja avaavat uusia mahdollisuuksia lääkehoidon kehittämiseen: TRPA1:n estäminen on lupaava uusi strategia lievittää tulehduksellisiin sairauksiin liittyvää tulehdusreaktiota sekä kipua.

Introduction

Imperfection is human. In fact, mankind, i.e. *Homo sapiens sapiens* species, and all individuals are imperfect in their own ways. On the larger scale, imperfection can be seen as fall of mighty kingdoms of the past or via our present-day inability to feed the world's population. On the other hand, smaller signs of our imperfections can be seen in our everyday lives e.g. our inability to draw an exact circle with a pen or forgetting our home keys on the kitchen table. Incomprehensibly, despite countless hours of revision by several experts of scientific text writing and technological aid provided by the achievements of world's cutting edge software engineers, this piece of academic text might still contain spelling mistakes. However, the flaws of man are perhaps one of the cardinal features that make human life interesting. Indeed, as the Chinese proverb says "Gold cannot be pure, and people cannot be perfect."

The immune system represents one very fascinating dimension of human biology. Meant to combat invading pathogens, the network of different tissues, cells and molecular cascades, over the course of evolution, has developed highly efficient, complex and extensive. In fact, 7% of our genes are dedicated to the immune system and there are dozens of different types and subtypes of immune cells, not to mention the myriad of signalling molecules and receptors (Kelley et al., 2005). The focus of this doctoral thesis is on a phenomenon called inflammation, an immune system driven reaction aimed at the clearance of the source of the disruption of the host tissue or system homeostasis. Inflammation is a crucial component of the immune system, which in turn, is vital for the survival of the human race. However, when inflammation is turned against the host, it gives rise to a major medical problem of our times: the inflammatory diseases. They are responsible for a major economic burden to mankind, but much more importantly, they cause tremendous individual suffering. Inflammatory diseases comprise classical conditions such as arthritis and asthma, but the perspective has broadened as many degenerative diseases, such as Alzheimer's disease, cardiovascular diseases and even obesity and obesity-related co-morbidities, have been shown to incorporate inflammatory features.

The immune system cannot act in a random manner. It should be able to unleash its inflammatory firepower on appropriate targets and to save the innocent ones, not

to speak of avoiding harming the host itself. Similarly, there are multiple mechanisms modulating the anti-inflammatory response in order to ensure the inflammatory reaction is kept in check. More importantly, a broad array of sensory systems have developed to fine-tune the immune response and inflammatory reaction. Pathogen recognition receptors such as toll-like receptors and NOD-like receptors detect external microbial threats and trigger inflammation to combat this threat. Furthermore, nociceptors are receptors which induce pain signals in order to provide the host with information about unfavorable events such as the presence of pathogenic bacteria or tissue damage and to further guide the body maintenance. There are also warning systems to sense the presence of harmful chemical compounds. One major family of these so-called chemoreceptors is the transient receptor potential ion channel family. One specific member of the family, transient receptor potential ankyrin 1 (TRPA1), a nociceptor and a chemosensor, has very recently become potentially associated with inflammation and therefore has drawn considerable interest. In fact, it also attracted my interest and the present Review of the Literature describes different aspects of the ion channel and further I will present results postulating that TRPA1 is an endogenous contributing factor in inflammatory states. (Kumar et al., 2010)

From the perspective of evolution, all traits in natural organisms, including the features of immune system and inflammation, are subject to the theory of cost-benefit trade-off. This means that each trait has its pros and cons and therefore is never completely perfect. However, in order that the trait should be favored by natural selection, the cost-benefit trade-off must be beneficial for the current environment. Human beings have developed an efficient immune system as an investment in body maintenance to protect against pathogens but as a downside, this has a negative impact on fertility, because a too vigorous immune system is a risk for fetal survival. It is a very delicate balance between proinflammatory traits, which protect from pathogens, and anti-inflammatory traits, which promote fertility; equilibrium is vital for the very existence of the species and its ability to adapt to the current living environment. (Van Bodegom et al., 2007; Westendorp et al., 2001)

Evolution takes time. If we survey how the human immune system has developed, we can appreciate that most of human evolution occurred in a fairly harsh environment. Thousands or perhaps even one hundred thousand years ago, human beings lived in very unhygienic conditions with little medical aid available. Formation of civilizations and cities enhanced the living conditions but provided also a good habitat for microbes and this may have even boosted the natural selection of so-

called pro-inflammatory individuals (Van Bodegom et al., 2007). However, during the past few centuries, man has become a master of niche construction, i.e. being able to alter the living environment to suit his/her needs (Laland & Brown, 2006). Mankind has made numerous inventions such as protective clothing and buildings, hygiene and modern medicine and overcome many deadly threats present in the environment. However, these changes have been far too rapid to allow evolution to keep pace with the new living environment. I find this as a very intriguing question; is our immune system unduly proinflammatory for our modern living environment and causing us more costs in the form of inflammatory diseases than benefits as a protective mechanism against invading pathogens? (Okin & Medzhitov, 2012)

In ancient times, an individual human normally lived about 40 or 50 years as hygiene, heating or penicillin did not exist. Nowadays, especially in the developed countries in the presence of these and many more amenities, human beings often live beyond 80 years. This rapid prolongation of the human lifespan has seen the emergence of many degenerative diseases, all of which have been recognized to display a strong inflammatory component. This is perhaps also due to the natural selection of proinflammatory traits, in that some of their costs only appear after the fertile age or even after the age of the naturally expected lifetime of our not-too-distant ancestors. This is an example of pleiotropic antagonism, a theory that explains how a single gene can control many traits; one can be beneficial but simultaneously an adverse trait may appear. Indeed, evolution rarely favors traits that benefit the individual after he/she passes the age of fertility and reproduction. Okin & Medzhitov and van Bodegom et al. have described this interesting aspect of the development of immune system and inflammation in their reviews (Okin & Medzhitov, 2012; Van Bodegom et al., 2007).

Is all the imperfection we see around us and especially in the mirror, only our view? Perhaps mankind has a good eye for detecting imperfection and a need for improvement? The urge to improve is indeed a very human feature, perhaps one of the key elements for development of mankind. In order to improve, we need to acquire knowledge and to understand. Human curiosity and respect for information have existed for millenia: the first libraries were founded over four thousand years ago and teachers and the wise have always enjoyed respect and approval. The present particle of the Latin verb *scire*, 'to know', is *sciens*, which means 'having knowledge' and the Latin *scientia* means 'knowledge'. Certainly, science is dedicated to increasing and treasuring our knowledge in various forms and should be used for the benefit of all mankind.

The driving force of this academic research has been curiosity and a passion for understanding the mechanisms of our immune system and inflammation. It focuses on TRPA1, a chemosensor which acts to trigger pain and inflammation when encountering harmful environmental compounds. However, I have examined the possibility that TRPA1 could turn against the body should it be activated in unsuitable occasions and as a consequence, promote detrimental inflammatory states. Ultimately, this research aims at providing new information, which could lead to the development of new strategies to treat inflammatory diseases, and in that way to lighten their burden of suffering.

1 Review of the Literature

1.1 Transient Receptor Potential Ion Channels

The observations made in 1969 by Cosens and Manning described a mutant Drosophila with transiently impaired vision after exposure to bright illumination. This finding set off a trail leading to the discovery of an ion channel superfamily greatly increasing our understanding of sensory physiology and many other body functions (Cosens & Manning, 1969). After electrophysiological recordings in Drosophila retina were conducted by Minke and co-workers in 1975, the mutant fruit fly was named as transient receptor potential (TRP) because the response to the intense light was only transient (Minke et al., 1975). The Drosophilia Trp gene was sequenced and cloned by Montell and Rubin in 1989 and it was found to display a structural similarity to many ion channels (Montell & Rubin, 1989). After only a few years, the TRP protein was identified as a light-sensitive ion channel permeable to Ca^{2+} (Hardie & Minke, 1992). The first mammalian ion channel with a sequence homology to the Drosophilia TRP, transient receptor potential canonical 1 (TRPC1), was identified in 1995 and soon many other mammalian TRP ion channels and channel families were discovered (Wes et al., 1995). It is now known that the original Drosophila TRP mutant had a loss-of-function mutation in the gene encoding the light-sensitive TRP ion channel, which is crucial for the sustainability of the phototransduction and together with its homologue TRP-like channel (TRPL) they made up the two light-sensitive ion channels critical for Drosophilia vision. Even though the Drosophila retinal function is distinct from its mammalian counterpart which does not include a TRP ion channel as a phototransductor, the original Drosophilia TRP has given its name and stimulated the discovery of the seven sub-families of the TRP ion channel superfamily, nowadays known to be crucial in physiological functions and many human diseases. (Hardie, 2011; Minke, 2010; Montell, 2011)

The TRP ion channel superfamily embraces at least 33 different ion channels divided into 7 subfamilies named ankyrin (TRPA), canonical (TRPC), melastatin (TRPM), mucolipin (TRPML), no mechanoreceptor potential C (TRPN), polycystin (TRPP) and vanilloid (TRPV). A total of 27 and at least one ion channel from each

subfamily is present in humans, except for the TRPN subfamily which is found only in non-mammalian species such as zebra fish and worms. Even though the superfamily is diverse with a multitude of functions and modalities, in general the membrane bound TRP ion channels function by mediating transmembrane cation flux according to the electrochemical gradient in order to depolarize the cell and pose their secondary effects. TRP ion channels are activated by diverse mechanisms including G protein-coupled receptor linked activation, ligand gating by a variety of endogenous and exogenous substances and mechanical, thermal and cell conformational coupling. The activity of different TRP ion channels is also modulated by a variety of mechanisms such as gene expression regulation and post-translational sensitizing, desensitizing and receptor trafficking. In structural terms, TRP ion channels resemble the voltage-gated ion channels and the TRP proteins are formed of six transmembrane domains S1-6 with a pore loop between S5 and S6 and contain an intracellular N- and C-terminal region with variable length and structure. These proteins are probably assembled into homo- and heterotetramers to form the functional ion channels. The further classification of the members of the 28 mammalian TRP ion channel superfamily and their functions are summarized in Table 1. (Nilius & Flockerzi, 2014; Ramsey et al., 2006; Schaefer, 2005)

Table 1. Mammalian TRP ion channels. The 28 distinct ion channels, their gating mechanisms and putative functions are given as described in the book Mammalian Transient Receptor Potential (TRP) Cation Channels (Nilius & Flockerzi, 2014). Abbreviations: adenosine diphosphate (ADP), G protein–coupled receptors (GPCR), phospholipase C (PLC), reactive nitrogen species (RNS), reactive oxygen species (ROS), phosphatidylinositol-4,5-bisphosphate (PIP_2)

Family	Ion channel	Ion permeability	Gating	Putative function
TRPA (ankyrin)				
	TRPA1	Ca^{2+}, Na^{+}, K^{+}, Mg^{2+}	Exogenous irritants, ROS, RNS and their metabolites	Nociception, neurogenic inflammation
TRPC (canonical)				
	TRPC1	Non-selective cation	PLC, store depletion, mechanical	Basic cell functions

	TRPC2	Ca^{2+}, non-selective cation	Diacylglycerol, erythropoietin	Erythropoietin signalling, reproductive functions
	TRPC3	Non-selective cation	Versatile	Versatile
	TRPC4	Ca^{2+}, Na^{+}, variable selectivity	Versatile; store depletion, GPCR	Versatile
	TRPC5	Ca^{2+}	PLC, store depletion, GPCR, ligands, cold	Neurotransmission, behavioural functions, smooth muscle functions
	TRPC6	Non-selective cation	Diacylglycerol	Smooth muscle contraction, slit diaphragm architechture
	TRPC7	Non-selective cation	PLC, diacylglycerol	Reproductive functions? breathing regulation?
TRPM (melastatin)				
	TRPM1	Non-selective cation	Light-induced protein interaction	Vision
	TRPM2	Ca^{2+}, Non-selective cation	ADP-ribose, Ca^{2+}, ROS, RNS	Release of vesicular mediators (cytokines, neurotransmitters etc.)
	TRPM3	Divalent cation	Non-specific ligands, noxious heat	Secretion of proinflammatory cytokines, release of insulin
	TRPM4	Non-selective cation	Ca^{2+}, PIP_2	Not well understood, smooth muscle and cardiac cell function?
	TRPM5	Na^{+}, K^{+}, Cs^{+}	Ca^{2+}, voltage	Taste, insulin secretion
	TRPM6	Ca^{2+}, Mg^{2+}, divalent cations	Insulin	Systemic Mg^{2+} homeostasis

	TRPM7	Divalent cation	Mg^{2+}, PLC, mechanical stimuli, low pH	Cellular and systemic Mg^{2+} homeostasis
	TRPM8	Non-selective cation	Cold, mechanical stimuli, various ligands	Cold nociception, pain, malignancies
TRPML (mucolipin)				
	TRPML1	Ca^{2+}, Fe^{2+}, Zn^{2+}, Na^{+}, K^{+}	Late endosome and lysosome associated factors	Membrane trafficking events
	TRPML2	Ca^{2+}, Na^{+}, Fe^{2+}	Endosome associated factors	Not well understood, calcium release from endolysosome?
	TRPML3	Na^{+}, K^{+}, Cs^{+}	PIP_2, synthetic ligands	Salty taste? pain?
TRPP (polycystin)				
	TRPP2	Ca^{2+}, non-selective cation	Ca^{2+}, pH	Complex cellular events, left-right asymmetry
	TRPP3	Ca^{2+}, non-selective cation	Voltage, alkalinisation, cell swelling	Unknown
	TRPP5	Ca^{2+}, non-selective cation	Unknown	Unknown
TRPV (vanilloid)				
	TRPV1	Non-selective cation, divalent > monovalent	Noxious heat, capsaicin	Nociception, thermoregulation, neurogenic inflammation
	TRPV2	Ca^{2+}, Mg^{2+}, Na^{+}, Cs^{+}, K^{+}	Heat, various ligands, mechanical stimuli	Nociception, thermal sensation, innate immunity, vascular tone

	TRPV3	Ca^{2+}, Na^{+}, Cs^{+}, K^{+}	Heat, various ligands	Skin functions
	TRPV4	Ca^{2+}, Mg^{2+}, non-selective cation	Moderate heat, mechanical and osmotic stimuli, various ligands	Osmo- and thermoregulation, mechanical sensation, nociception, neurogenic inflammation
	TRPV5	Ca^{2+}	Constitutively active	Ca^{2+} reabsorbtion and homeostasis
	TRPV6	Ca^{2+}	Constitutively active	Ca^{2+} homeostasis

1.2 TRPA1

The main target of interest in this thesis project is TRPA1, which is the only known member of the TRPA subfamily found in mammals. TRPA1 was first discovered by Daniel Jaquemar and colleagues in 1999 and it was reported to have 18 repeats that are related to the cytoskeletal protein ankyrin in its N-terminus and to be structurally related to the TRP ion channels (Jaquemar et al., 1999). Within a few years, the protein was named ankyrin-like with transmembrane domains 1 (ANKTM1) and found to be mainly expressed in sensory neurons but soon it was renamed as TRPA1 and the crucial function of TRPA1 to sense noxious environmental compounds was discovered (Bandell et al., 2004; Jordt et al., 2004; Story et al., 2003). It is now appreciated that TRPA1 is a ligand-gated and membrane bound cation channel of 120-130 kD. Furthermore, TRPA1 is known to function not only as a chemosensor of environmental potentially harmful compounds but also as a nociceptor and be involved in neurogenic inflammation by releasing proinflammatory neuropeptides such as substance P, calcitonin gene-related peptide (CGRP) and neurokinin A (Nassini et al., 2014; Nilius et al., 2012).

1.2.1 Trpa1 Gene and Protein Structure

The human gene encoding TRPA1 is named trpa1 and it is composed of 27 exons and 55 701 base pairs located in human chromosome 8q13 (Nilius et al., 2012). Many other species also have a TRPA1 homologue and evidently the ability of TRPA1 to

sense electrophilic compounds has been conserved for approximately 500 million years (Kang et al., 2010). The TRPA1 protein is formed of roughly 1 100 amino acid with a molecular weight of 120-130 kD and its structure is very similar to the other TRP channels. It is composed of six transmembrane domains S1-6; between the domains S5 and S6 is the putative ion permeable pore estimated to be 1.10 nm in diameter (Karashima et al., 2010; Nilius et al., 2011). The long cytoplasmic N-terminus contains a special feature of TRPA1, the 14-18 ankyrin repeat domains which have also provided TRPA1 with its name. The exact role of the ankyrin repeat domains is still a topic of speculation, but truncation of the motif has been shown to result in the incomplete transition of the channel to the plasma membrane; there

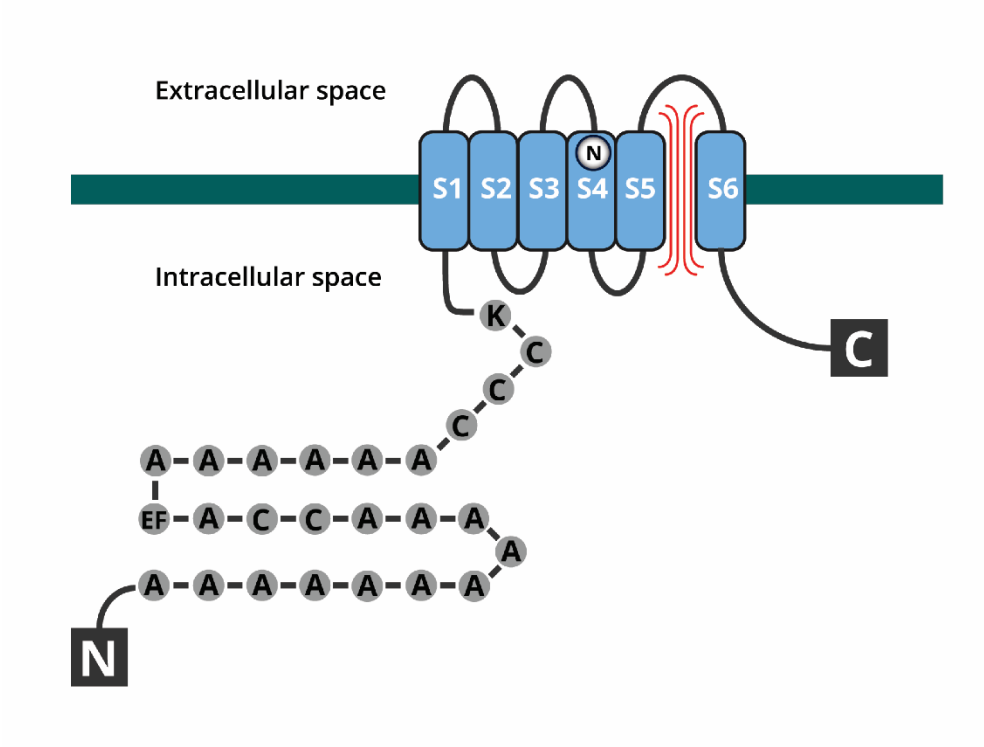


Figure 1. TRPA1 ion channel protein structure. The six transmembrane domains (S1-S6) are located at cell membrane and the ion permeable pore is located between S5 and S6. Within the S4 domain locates the Asn855 (N), which is the site for the gain-of-function mutation which causes familial episodic pain syndrome. The N- and C-domains are located intracellularly and the long N-terminus contains the ankyrin repeat domains (A), reactive cysteine (C) and lysine (K) residues important for the channel activation by reactive compounds and the calcium responsible EF hand motif (EF). Contents design by Lauri Moilanen and graphical design by Joonas Mykkänen.

is also data indicating that the ankyrin repeat domains contribute to the changes in the protein conformation associated with ligand binding (Nilius et al., 2011; Zayats et al., 2013). Strikingly, however, a recent study showed TRPA1 could be correctly folded and functionally intact even if the ankyrin repeat had been genetically removed (Moparthi et al., 2014). An EF hand motif is located also within the ankyrin repeat domains, providing a target of interaction for intracellular Ca^{2+} which is also known to regulate activation of the Ca^{2+} permeable TRPA1 (Doerner et al., 2007; Nilius et al., 2011). Another important structure of the N-terminus of TRPA1 protein is the section of cysteine residues. This section is crucial for the activation of TRPA1 by covalent modification by many electrophilic compounds but interestingly, the cysteine residues also provide a site for desensitization (Ibarra & Blair, 2013; Macpherson et al., 2007). Furthermore, as TRPA1 is mainly organized in homotetramers, an electron microscopic study of TRPA1 has proposed that the cysteine residues facing the neighbouring subunits form a ligand-binding pocket, enabling the formation of disulfide bonds between the cysteine residues and thereby contributing to the channel gating (L. Wang et al., 2012). A putative illustration of the TRPA1 protein structure is presented in Figure 1.

1.2.2 TRPA1 Expression

TRPA1 was originally found in cultured fibroblasts but the understanding on the role of TRPA1 in nociception and chemosensation was advanced when its neuronal expression was appreciated (Jaquemar et al., 1999; Story et al., 2003). TRPA1 is mainly expressed in sensory terminals and dorsal root, trigeminal and nodose ganglia of small myelinated Aδ- and unmyelinated C-fiber primary sensory peptidergic and non-peptidergic neurons. Interestingly, TRPA1 frequently co-localizes with its sibling ion channel, TRPV1 (Nilius et al., 2011; Zygmunt & Högestätt, 2014). The expression of TRPA1 is not limited to neurons since TRPA1 is found in many other cell types spread throughout the body. TRPA1 expression has been detected in cell types such as epithelial cells, melanocytes, mast cells, odontoblasts, urothelium, enterochromaffin cells, β-cells of the Langerhan's islets, synoviocytes, and endothelial cells (Fernandes et al., 2012; Nassini et al., 2014; Zygmunt & Högestätt, 2014).

TRPA1 expression is also prone to gene expression alterations and especially the upregulation of TRPA1 has been shown to be mediated by several factors. In dorsal root ganglia, TRPA1 expression has been shown to be increased by activation of p38

mitogen-activated protein kinase, by interleukin-6 (IL-6) signal transducer gp130, by nerve growth factor, by the neuronal survival factor, artemin, and strikingly by a well-known direct TRPA1 activator, mustard oil (Diogenes et al., 2007; Elitt et al., 2006; Malsch et al., 2014; Obata et al., 2005; Schmidt et al., 2009). In addition, TRPA1 gene expression has been reported to be upregulated in cultured synoviocytes by tumor necrosis factor-α (TNF-α) and IL-1α-induced nuclear factor-κB (NF-κB) signalling, leading to activation of hypoxia-inducible factor-1α (Hatano et al., 2012). In mast cells and in neurons derived from dorsal root ganglia and dermal afferents, the expression of TRPA1 is increased in experimental atopic dermatitis and also by IL-13 stimulation (Oh et al., 2013). Furthermore, TRPA1 expression has also been shown to be upregulated by a still unrecognized molecular mechanism in experimental nerve injury in both dorsal root ganglia and bladder and in experimental acute pancreatitis (Andrade et al., 2012; Frederick et al., 2007; Schwartz et al., 2011). Different transcription factors have been found to have a putative binding site in the human Trpa1 gene promoter and are detailed in Table 2, even though their functional significance is still largely undiscovered.

Table 2. Trpa1 gene transcription factors. The identified transcription factors found to have a binding site in the human Trpa1 gene promoter as presented by The GeneCards Human Gene Database (www.genecards.com)

ARP-1, AhR, Arnt, C/EBPα, C/EBPβ, CUTL1, E47, ER-α, Evi-1, FOXI1, HFH-3, ITF-2, LHX3a/Lhx3a, LHX3b/Lhx3b, NF-AT, NF-κB, Nkx6-1, POU2F1, POU2F1a, POU3F2, POU6F1, PPAR-γ1, PPAR-γ2, Pax-4a, RelA, S8, Tal-1β, c-Rel, p53

1.2.3 TRPA1 Electrophysiology

TRPA1 is classified as a non-selective cation channel permeable to both monovalent and divalent ions and it is characterized by its relatively high conductance. TRPA1 has a single channel conductance of 40-180 pS depending on the stimuli and measurement conditions (Zygmunt & Högestätt, 2014). Many functions induced by TRPA1 activation are frequently explained by the influx of Ca^{2+} ions and TRPA1 has a high Ca^{2+} permeability PCa/PNa ranging from 0.84 up to 7.9 contributing up to 23% of the inward TRPA1 current (Karashima et al., 2010; Nilius et al., 2011; Story et al., 2003). Interestingly, both intra- and extracellular Ca^{2+} concentrations have complex regulatory effects on TRPA1 activation and channel conductance properties, which still are not fully understood. The extracellular Ca^{2+} concentration

may affect the pore size of TRPA1 and therefore potentially affect the channel conductance as an increase in extracellular Ca^{2+} concentration has been shown to contract the pore and vice versa (Banke et al., 2010). Furthermore, the concentration of extracellular Ca^{2+} regulates the velocity and duration of the activation of TRPA1 and a high extracellular Ca^{2+} concentration has been shown to totally abolish the activation capacity of TRPA1 (Nilius et al., 2011; Y. Y. Wang et al., 2008). The intracellular Ca^{2+} concentration, possibly by binding to N-terminal EF hand motifs, has an activator property on TRPA1 at low concentrations, but high concentrations caused specifically by the influx of Ca^{2+} through TRPA1 suppress the channel (Nilius et al., 2011; Y. Y. Wang et al., 2008).

1.2.4 Exogenous Chemical Activators of TRPA1

The role of TRPA1 as a chemosensor is supported by the fact that a plethora of exogenous compounds are activators of TRPA1. Many TRPA1 openers are recognized to be chemically electrophilic compounds (i.e. substances receptive for eletrons) and they exert their TRPA1 gating by covalent modification of the cysteine and lysine residues in the N-terminal region of TRPA1 (Bang & Hwang, 2009; Hinman et al., 2006; Macpherson et al., 2007). For example, the pungent compound of mustard oil, i.e. allyl isothiocyanate (AITC), has been long used to induce neurogenic inflammation and to activate the release of proinflammatory neuropeptides (Louis et al., 1989). However, it was only 10 years ago when its exact mechanism of action was discovered and it was noted that AITC was one of the most robust activators of TRPA1 (Jordt et al., 2004). Nowadays AITC is used frequently as a research tool to study activation of TRPA1. Furthermore, there are other notable exogenous pungent compounds known to activate TRPA1 e.g. cinnamaldehyde found in cinnamon, allicin found in garlic, acrolein found in exhaust fumes and tobacco smoke and morphanthridine analogues found in potent tear gases (Bandell et al., 2004; Bautista et al., 2006; Brone et al., 2008; Macpherson et al., 2005). Interestingly, some of electrophilic and non-electrophilic compounds, such as cinnamaldehyde, camphor and apomorphine, exert a bimodal effect on TRPA1: at low concentrations they elicit a dose-dependent activation of TRPA1 but at high concentrations, their effect is inhibitory (Alpizar et al., 2013; Schulze et al., 2013).

In addition to the many electrophilic TRPA1 activators, there are a variety of non-electrophilic activators and their mechanism of action also differs from the electrophilic agents. Some non-electrophilic TRPA1 activators have been proposed

to exert a direct interaction with the TRPA1 protein but much still remains unclear (Zygmunt & Högestätt, 2014). Interestingly, an intravenous anesthetic, propofol, has been identified as a non-electrophilic activator of TRPA1 and this effect could explain the pain induced by application of propofol observed in clinical practice (Fischer et al., 2010). In addition, many components of marijuana are known to activate TRPA1. The psychoactive compound, Δ^9-tetrahydrocannabinol and the non-psychoactive cannabidiol and cannabichromene all have been reported to activate TRPA1 even though they also have potential to activate other TRP ion channels (De Petrocellis et al., 2008; Jordt et al., 2004). Some important TRPA1 activators are presented in the Table 3.

Table 3. TRPA1 activators. A list of some important exogenous and endogenous TRPA1 activators as presented by The British Pharmacological Society and the International Union of Basic and Clinical Pharmacology database (www.guidetopharmacology.org).

Substance	**Source**	**EC_{50} (studied species)**
Acetaldehyde	Exogenous	79.43 μM (human)
Acrolein	Exogenous	5.01 μM (human)
Allyl isothiocyanate	Exogenous	19.95 μM (mouse)
Cinnamaldehyde	Exogenous	63.10 μM (mouse)
Formalin	Exogenous	398.10 μM (mouse)
Nicotine	Exogenous	15.85 μM (human)
Δ^9-tetrahydrocannabinol	Exogenous	12.59 μM (human)
4-hydroxynonenal	Endogenous	25.12 μM (rat)
4-oxononenal	Endogenous	2.00 μM (mouse)
H_2O_2	Endogenous	251.19 μM (mouse)
Methylglyoxal	Endogenous	1.0 μM (human)
NaHS	Endogenous	1258.93 μM (mouse)
Prostaglandin A_2	Endogenous	25.12 μM (mouse)

1.2.5 Endogenous Chemical Activators of TRPA1

Perhaps one of the most interesting properties of TRPA1 is its ability to sense endogenous painful and proinflammatory compounds generated mainly under inflammatory conditions and tissue injury. Indeed, TRPA1 is endogenously targeted by many reactive oxygen and nitrogen species (ROS and RNS) and their metabolites. ROS such as H_2O_2, hypochlorite and superoxide have been shown to activate TRPA1; their mechanism of action is most likely through a cysteine oxidation or disulfide formation (Bessac et al., 2008; Takahashi & Mori, 2011). With respect to the RNS, such as nitric oxide (NO), the mechanism of action is explained as S-nitrosylation (Takahashi et al., 2008; Takahashi & Mori, 2011). In addition to direct effects on TRPA1, since ROS and RNS are highly reactive, they can react with unsaturated fatty acids in cell membranes to generate substances such as 4-hydroxynonenal, 4-hydroxyhexenal, 4-oxononenal and nitrooleic acid which are also major activators of TRPA1 (Andersson et al., 2008; Taylor-Clark et al., 2009; Trevisani et al., 2007). Prostaglandins are a large group of endogenous signalling molecules and many of them are proinflammatory. Interestingly, at least two electrophilic cyclopentone prostaglandins, 15-deoxy-δ12,14-prostaglandin J_2 and prostaglandin A_2, have been identified as endogenous TRPA1 activators probably through a direct interaction with the ion channel (Taylor-Clark et al., 2008). A decline in extracellular pH is involved in the micro-environment of local inflammation, ischemia and tissue damage and intriguingly, pH levels of 7 and lower, have a potency to activate specifically human TRPA1 in a proton concentration-dependent manner (de la Roche et al., 2013; Takahashi et al., 2008). Some important TRPA1 activators are presented in Table 3.

In addition to rather direct coupling by endogenous ligands, TRPA1 is also modified by endogenous cellular mechanisms. G protein-coupled receptors, of which at least bradykinin receptor and protease-activated receptor-2 have been identified, may sensitize TRPA1 through secondary mediators such as protein kinase A (PKA) and phospholipase C (PLC). These intracellular signalling regulators have been proposed to lead to phosphorylation of TRPA1 and thereby sensitize it towards its activation by other ligands, such as AITC and also to induce the trafficking of TRPA1 to the cell membrane (Bandell et al., 2004; Dai et al., 2007; Schmidt et al., 2009; S. Wang et al., 2008). However, data concerning phosphatidylinositol-4,5-bisphosphate (PIP_2), the main substrate cleaved by activated PLC, is somewhat discordant. Even though the activation of PLC, resulting in a decrease in the levels of PIP_2, has been shown to sensitize TRPA1 and that the direct application of PIP_2

to the studied cells has been reported to inhibit the TRPA1 activation, there is also some data indicating that an increase in the PIP_2 concentrations delays the desensitization of TRPA1 and that application of a PIP_2 scavenger evokes an accelerated desensitization of the ion channel (Karashima et al., 2008; D. Kim et al., 2008; S. Wang et al., 2008). Interestingly, TRPA1 is commonly co-localized with TRPV1 and these two channels can form heterotetramers (Fischer et al., 2014). TRPV1 can also interact with TRPA1 and modulate its activity (Patil et al., 2010).

1.2.6 Activation of TRPA1 by Cold and Mechanical Stimuli

Initially, TRPA1 has been described to be activated by cold temperatures below 10°C (Story et al., 2003). However, this has been actively debated and there is considerable experimental evidence failing to demonstrate any direct cold-induced TRPA1 activation (Caspani & Heppenstall, 2009; Cordero-Morales et al., 2011; Jordt et al., 2004; Zurborg et al., 2007). Since most experiments investigating the activation of TRPA1 have been performed either in rodent dorsal root ganglion neurons or cultured HEK 293 cells transfected with human TRPA1, the possible explanation for inconsistent data concerning the cold-induced activation of TRPA1 could be species differences as proposed in a recent study (Chen et al., 2013). It is also possible that TRPA1 does not play a major role in physiological cold sensation but is indeed involved in noxious cold sensation or the hypersensitivity associated with other pathological conditions as will be discussed in more detail later (Obata et al., 2005).

Many TRP channels are mechanosensitive and this has been proposed also as a mechanism to explain TRPA1 activation (Pedersen et al., 2005). *In vitro*, the application of a hypertonic solution to the extracellular space induces cell shrinkage and this can be used to study the effects of mechanical stimuli on the cell. Indeed, application of hypertonic solution to TRPA1 expressing cells induces Ca^{2+} currents not seen in cells treated with a TRPA1 blocker, demonstrating the importance of TRPA1 in mechanosensation *in vitro* (X. F. Zhang et al., 2008). Furthermore, TRPA1 has proven to be mechanosensitive also *in vivo*. In line with results observed after pharmacological TRPA1 inhibition, mice lacking TRPA1 display lower neuron action potential firing rates in response to mechanical stimulation although this effect may be confined to only a specific subset of nociceptors (Kerstein et al., 2009; Kwan et al., 2009). In addition, the role of TRPA1 in mechanical hyperalgesia induced by external stimuli, such as Complete Freund's Adjuvant or hydrogen sulfide, have been defined (da Costa et al., 2010; Okubo et al., 2012).

1.2.7 Blockers of TRPA1

Due to the pivotal role of TRPA1 in many adverse effects such as pain and inflammation, TRPA1 has attracted increasing interest as a drug target. Problems have occurred in the differences between species, as compounds may have different effects on human and rodent TRPA1, but the ion channel has also been a rather challenging molecular target. In contrast to TRPA1 activators, there are much fewer compounds with the ability to inhibit TRPA1 and most of them have been purposely developed. (Nassini et al., 2014)

The first selective TRPA1 blocker, HC-030031, was developed by Hydra Biosciences in 2007 (McNamara et al., 2007). Chemically HC-030031 contains a xanthine alkaloid core and it binds to TRPA1 reversibly inhibiting its activation (McNamara et al., 2007). HC-030031 is still one of the most widely used pharmacological research tool to inhibit TRPA1 both *in vitro* and *in vivo* and it was used also in many of our studies. Further development of HC-030031 led to the synthesis of a structurally related compound, TCS 5861528 (also known as Chembridge-5861528) and this compound has also been investigated both *in vivo* and *in vitro* (Wei et al., 2009; Wei, Chapman et al., 2010). Subsequently, during 2007 Abbott discovered an oxime derivate, AP18, which after further developmental work led to A-967079 (Chen et al., 2011; Petrus et al., 2007). In contrast to HC-030031, A-967079 inhibits TRPA1 by binding inside the pore vestibule of the ion channel (Klement et al., 2013). Some non-selective TRP ion channel blockers, such as ruthenium red, have also efficacy at inhibiting TRPA1 (Nagata et al., 2005). Furthermore, TRPA1 is most likely inhibited by some naturally occurring polyphenols e.g. resveratrol and gallic acid (Trevisan, Rossato et al., 2014; Yu et al., 2013).

A breakthrough in the research into TRPA1 blocking drugs for clinical use emerged in 2011 when Glenmark Pharmaceuticals Ltd. announced that its TRPA1 blocker, GRC 17536, had entered phase 2a double blind placebo controlled clinical trial for neuropathic pain, asthma and chronic obstructive pulmonary disease (*Glenmark's novel molecule 'GRC 17536' for pain and respiratory entering human trials,* 2011). The latest press release from 2014 reported positive data in the proof of concept study and further news are being eagerly awaited (*Glenmark's TRPA1 antagonist 'GRC 17536' shows positive data in a proof of concept study,* 2014). Scientific studies have also proven GRC 17536 to be a potential blocker of TRPA1 *in vitro* and *in vivo* (Mukhopadhyay et al., 2011; Mukhopadhyay et al., 2014). Recently, a Finnish pharmaceutical corporation Orion Pharma reported the start of clinical phase 1 trial

on their TRPA1 blocker ODM-108 for the treatment of neuropathic pain (*Orion Group Interim Report January-March 2015*).

1.2.8 Physiological Function of TRPA1

The physiological function of TRPA1 seems to be chemo- and mechanosensation and possibly thermosensation of cold. TRPA1 might also be involved in the regulation of cardiovascular system, pancreas and bladder function.

As already discussed, TRPA1 is highly sensitive to many external noxious compounds. Located primarily in the sensory nerve endings of small myelinated Aδ- and unmyelinated C-fibers, TRPA1 transmits a noxious sensation to the central nervous system aiming at avoidance of the substance encountered. Indeed, many substances that cause a noxious sensation, such as AITC in mustard oil, piperine in black pepper and diallyl disulfide in garlic, are robust TRPA1 activators (Bautista et al., 2005; Jordt et al., 2004; Okumura et al., 2010). An interesting example of TRPA1 activation is the case of the "headache tree". The California bay laurel or *Umbellularia californica* is known to cause severe headaches if an individual inhales its fumes. The active compound has been identified as umbellulone but the mechanism of action of umbellulone was unknown until 2011 when Nassini and co-workers reported that experimental application of umbellulone induced a significant TRPA1 activation and this led to the release of CGRP and meningeal vasodilation and subsequently headache (Nassini et al., 2012).

The concept of TRPA1 in mechanosensation was originally introduced in the hair cells of the inner ear and a role for TRPA1 in hearing was hypothesized (Corey et al., 2004). However, the current opinion in mammals is that TRPA1 does not have any role in auditory transduction since it has been demonstrated that TRPA1 deficient mice exhibit normal hearing (Bautista et al., 2006). Nonetheless, as discussed earlier, there is a body of evidence proposing a role for TRPA1 in physiological and pathophysiological (i.e. allodynia and hyperalgesia) mechanotransduction pathway (Corey et al., 2004; Kerstein et al., 2009; Kwan et al., 2009; Tsutsumi et al., 2013; X. F. Zhang et al., 2008). Conclusive understanding of this topic will still require more research.

As discussed, TRPA1 might be activated by cold temperatures but a role in cold allodynia is more likely (Obata et al., 2005; Story et al., 2003). Furthermore, cold temperature might sensitize TRPA1 to allow its activation by other ligands and

thereby have a regulatory role on the channel activity (del Camino et al., 2010). Importantly, TRPA1 does not seem to have a role in systemic thermoregulation which is essential for tolerance to TRPA1 blockers (Chen et al., 2013; Chen et al., 2011; de Oliveira et al., 2014). The case for TRPV1 was different and the drug candidates inhibiting TRPV1 have failed to make clinical breakthroughs, mainly due to adverse effects including the appearance of hyperthermia and attenuated sensation for noxious heat (Brederson et al., 2013).

In the cardiovascular system, some studies have indicated a role for TRPA1 in vasodilation and increasing cardiac output (Earley, 2012). The application of an external TRPA1 activator has been shown to induce vasodilation and an increase in peripheral blood flow in a CGRP-dependent manner, suggesting that this is a neuronal effect (Graepel et al., 2011; Pozsgai et al., 2010). Furthermore, the possible role of TRPA1 in the myoendothelial junction, an anatomical microstructure mediating signalling between endothelial and lining smooth muscle cells, has also been studied. The findings indicate that TRPA1 activation in endothelial cells resulting in Ca^{2+} influx into the endothelial cells could mediate hyperpolarization of the smooth muscle cells and thereby induce vasodilation (Earley et al., 2009). The systemic intravenous administration of TRPA1 activator cinnamaldehyde has been shown to exert bimodal effects in mice, initially causing a short-term fall in mean arterial pressure and heart rate followed by a sustained rise in both parameters. However, in anesthetized TRPA1 deficient mice, the resting mean arterial pressure and heart rate were similar to the values measured in the corresponding wild type mice (Pozsgai et al., 2010).

In pancreas, TRPA1 is expressed in the β-cells of the Langerhan's islets responsible for insulin production. In these cells, TRPA1 activation synergistically with KATP and L-type voltage-gated Ca^{2+} ion channels seems to facilitate insulin secretion (Cao et al., 2012; Numazawa et al., 2012). Methylglyoxal is a substrate produced in hyperglycemic conditions. Interestingly, it has been shown to be a powerful endogenous TRPA1 activator and therefore hypothesized to contribute to pancreatic insulin secretion regulation (Cao et al., 2012). In addition, the accumulation of methylglyoxal to sensory neurons has been emphasized in diabetic neuropathy and the role of TRPA1 activation in this process has been proposed (Eberhardt et al., 2012; Koivisto et al., 2012).

TRPA1 is co-localized with TRPV1 in nerve fibres across the bladder in urothelium, suburothelial space and muscle layer, as well as around blood vessels but also in urothelial cells (Streng et al., 2008). Up to 51% of the primary sensory nerves

innervating the bladder, were positively immunoreactive for TRPA1 staining and the highest expression was found in dorsal root ganglia (Du et al., 2007). In functional studies, application of AITC or cinnamaldehyde in the rat bladder increases the bladder contraction and voiding frequency and decreases the voiding volume (Du et al., 2007; Streng et al., 2008). It has been also speculated that TRPA1 activation possibly by H_2S, could contribute to the overactive bladder associated with cystitis (Skryma et al., 2011). However, the role of TRPA1 in cardiovascular, pancreas and bladder function is not conclusive and requires more research.

1.2.9 Non-Neuronal TRPA1

Even though most of the research on TRPA1 has focused on its function in sensory neurons, there is abundant expression of TRPA1 in some non-neuronal cells. Many of these non-neuronal cell types contribute in sensory and regulatory functions and frequently are located in lining tissues. These cell types and their putative functions have been partly discussed earlier and are summarized in Table 4.

1.2.10 TRPA1 in Pain

After reports were published establishing the role of TRPA1 in cold- and chemically-induced nociception, extensive research on the role of TRPA1 in pain has been carried out (Bandell et al., 2004; Bautista et al., 2013; Story et al., 2003). Direct or indirect activation of TRPA1 has been shown to elicit spontaneous pain, hyperalgesia and allodynia both in acute and persistent study settings (Bandell et al., 2004; da Costa et al., 2010; Obata et al., 2005; Wei et al., 2011; Wei et al., 2012). The pain evoking properties of TRPA1 openers are not limited to rodents but also healthy human volunteers respond to cinnamaldehyde by spontaneous pain and hyperalgesia (Namer et al., 2005; Olsen et al., 2014).

Acute inflammation is orchestrated by the release of various factors and this total environment is sometimes referred to as the "inflammatory soup". As discussed earlier, many factors associated with inflammation, particularly ROS and RNS and their metabolites, can activate TRPA1 and thereby contribute to the development of inflammatory pain (Andersson et al., 2008; Graepel et al., 2011; Taylor-Clark et al., 2009; Yoshida et al., 2006). Activation of TRPA1 leads also to a feed-forward mechanism, in which the activated sensory nerve terminal releases proinflammatory neuropeptides such as substance P and CGRP which drive the inflammation and

nociception onwards and induce further local hyperalgesia (Bandell et al., 2004; Nassini et al., 2014). There is now a body of evidence indicating that treatment with a TRPA1 blocker or genetic deficiency of the ion channel can inhibit acute pain and hyperalgesia induced by direct TRPA1 activators or proinflammatory compounds that increase the production of ROS and RNS (Eid et al., 2008; Fernandes et al., 2011; McNamara et al., 2007; Petrus et al., 2007). Acute inflammation can cause

Table 4. Non-neuronal expression of TRPA1

Tissue	Cell Type	Putative Function	References
Inner ear	Hair cells	Mechanosensation	(Corey et al., 2004)
Vasculature	Endothelial cells	Vasodilation	(Earley et al., 2009)
Pancreas	β-cells	Insulin secretion	(Cao et al., 2012; Numazawa et al., 2012)
Gastrointestinal tract	Enterochromaffin cells	Gastrointestinal motility	(Nozawa et al., 2009)
Skin	Keratinocytes, fibroblasts and melanocytes	Promotion of erythema and production of proinflammatory factors	(Atoyan et al., 2009; Jain et al., 2011)
Teeth	Dental pulp fibroblasts	Cold sensation	(Y. S. Kim et al., 2012)
Lungs	Airway and lung fibroblasts and epithelial cells	Production of proinflammatory factors	(Mukhopadhyay et al., 2011)
Bladder	Urothelial cells	Bladder contractibility	(Streng et al., 2008)
Synovial membrane	Synoviocytes	Regulation of proinflammatory factors	(Hatano et al., 2012; Kochukov et al., 2006)
Immune system	Mast cells	Itch and production of proinflammatory factors	(Oh et al., 2013)

a prolonged hyperalgesia lasting for weeks after the initial stimuli and furthermore, mice lacking TRPA1 develop a reduced extended hyperalgesia in experimental inflammation suggesting that activation of TRPA1 has a role in the development and/or maintenance of hyperalgesia (Fernandes et al., 2011; Garrison & Stucky, 2014).

The role of TRPA1 has been widely studied in neuropathic pain, in which nociception is induced by damage or dysfunction of the nervous system in different ways such as substance-induced or idiopathic neuropathy or nerve injury. In experimental models, ligation of lumbar spinal nerve has induced a long-lasting cold hyperalgesia but also increased the expression of TRPA1 in the neighbouring superior dorsal root ganglia, which largely contribute to the neuropathic pain and hypersensitivity associated with the ligation of the adjacent inferior lumbar spinal nerve (Gold, 2000; Obata et al., 2005). Interestingly, inhibition of TRPA1 by systemic or intrathecal drug treatment or by genetic depletion largely decreased the cold and mechanical hyperalgesia induced by the stimuli such as nerve injury or application of AITC or Complete Freund's Adjuvant (Chen et al., 2011; Eid et al., 2008; Katsura et al., 2006). Even though only few clinically significant TRPA1 mutations in human have been identified, a specific gain-of-function mutation of TRPA1 has been claimed to be responsible for a rare familial episodic pain syndrome. The patients carrying this mutation of TRPA1 suffer from episodes of severe upper body pain triggered by fasting and physical stress. Electrophysiological examination of the mutated channel revealed that it displayed a normal pharmacological profile to ligand binding but an increased current flow through the activated channel at negative membrane potentials. (Kremeyer et al., 2010)

The functional location of TRPA1 e.g. in TRPA1-induced chemosensation is often described in the peripheral end of the primary sensory nerve ending. Tactile allodynia, i.e. a state in which an innocuous mechanical stimulus evokes pain, can be induced by both peripheral and central sensitization (Julius & Basbaum, 2001). Furthermore, TRPA1 has been associated with the central mechanism underpinning tactile allodynia (Koivisto et al., 2014). Indeed, both systemic and intrathecal treatment with a TRPA1 blocker is effective in inhibiting the tactile allodynia induced by the peripheral neuropathy associated with diabetes mellitus, although peripheral dosing of the TRPA1 blocker has a much weaker effect (Koivisto et al., 2012; Wei, Koivisto et al., 2010). However, it appears that peripheral TRPA1 is not a low threshold mechanosensor responsible for tactile allodynia (Kerstein et al., 2009). Therefore, it is likely that in the case of tactile allodynia, the centrally located TRPA1

plays a major role in the central sensitization to peripherally evoked mechanical stimuli to induce noxious sensation.

Nerve growth factor has been associated with acute and sustained pain and hyperalgesia in many conditions such as cancer (McKelvey et al., 2013). In addition, nerve growth factor has been shown to increase the expression of TRPA1 and to enhance pain and hyperalgesia induced by TRPA1 openers also in studies conducted in man (Diogenes et al., 2007; Weinkauf et al., 2014). Furthermore, blocking the nerve growth factor has shown positive results in attenuating tumor cell proliferation and the pain associated with cancer and importantly the expression of TRPA1 was also down-regulated (Ye et al., 2011). Although much still remains to be discovered, TRPA1 could play a role in cancer pain and could therefore provide a target to treat pain, at least in some cancer types.

Some chemotherapeutic agents used to treat cancer, such as platinum-based oxaliplatin and cisplatin, are known to induce difficult peripheral neuropathic pain as common adverse effects. Indirect TRPA1 activation by oxaliplatin and cisplatin has been reported in nerve endings likely via generation of ROS (Nassini et al., 2011). In addition, prolonged hypersensitivity induced by injection of oxaliplatin or cisplatin can be transiently reversed by pharmacologically blocking TRPA1 and the hypersensitivity is abolished in TRPA1 deficient mice (Nassini et al., 2011; Nativi et al., 2013). Interestingly, systemic treatment with oxaliplatin also sensitizes mice to the nociception induced by local application of a TRPA1 channel opener (Zhao et al., 2012).

The link between severe headache due to migraine and TRPA1 has emerged during the past years. It is known that trigeminal neurons play a major part in the pathogenesis of migraine and their ability to cause vasodilation in meningeal vessels via CGRP has been highlighted as a possible cause of migraine (Bergerot et al., 2006). Recently, it has been shown that experimental application of a TRPA1 channel opener to the nasal mucosa or by inhalation induced a TRPA1-dependent release of CGRP leading to vasodilation in meningeal vessels (Kunkler et al., 2011; Kunkler et al., 2015). Furthermore, many substances, such as acrolein or formaldehyde, which have been associated with the onset of migraine or cluster headache have been found to activate TRPA1 (Nassini et al., 2014). Hence, bearing in mind the findings on the headache tree described above, it is possible that TRPA1 activation plays a role in migraine onset and headache. Therefore, TRPA1 blockers could prove useful in treating headache.

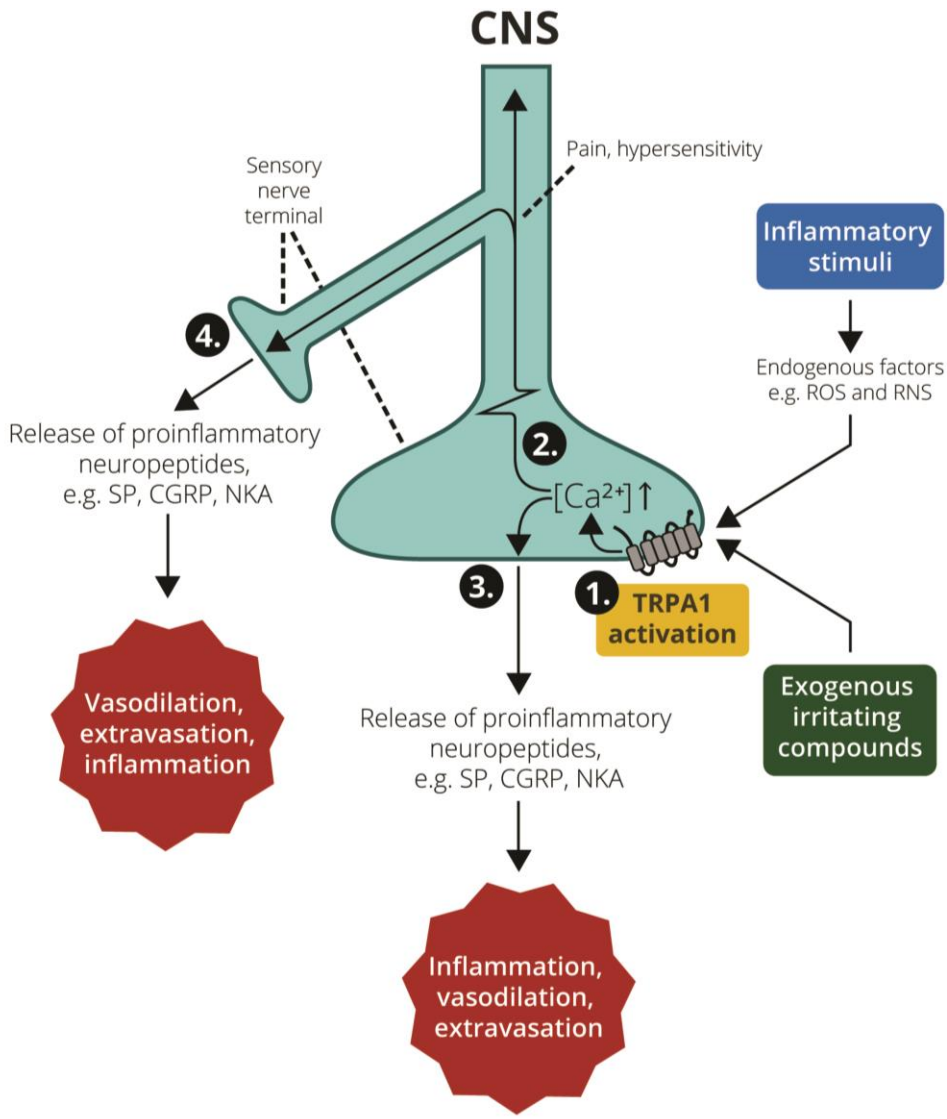


Figure 2. **TRPA1 in neurogenic inflammation.** 1. TRPA1 located on the terminal end of the sensory neuron, is activated by a direct external irritating compound (such as allyl isothiocyanate, AITC) or endogenously formed inflammatory factors (such as reactive oxygen and nitrogen species, ROS and RNS). 2. Activation of TRPA1 leads to influx of Ca^{2+} ions and depolarization of the terminus. This exceeds the firing threshold and nociceptive nerve signal is transmitted towards the central nervous system (CNS). 3. The activated nerve terminal releases proinflammatory neuropeptides substance P (SP), calcitonin gene-related peptide (CGRP) and neurokinin A (NKA), which promote local inflammation. 4. The nerve impulse propagates antidromically towards a nearby sensory terminus of the same neuron to release proinflammatory neuropeptides SP, CGRP and NKA which elicit proinflammatory effects especially to vessels feeding the original area of inflammation. Contents design by Lauri Moilanen and graphical design by Joonas Mykkänen.

1.2.11 TRPA1 in Inflammation

The early findings showed that in addition to nociception, direct activators of TRPA1 were able to induce an acute inflammatory response (Bautista et al., 2006). Curiously, long before its recognition as a potent TRPA1 activator, AITC had been used to trigger neurogenic inflammation and to induce the release of two proinflammatory neuropeptides, substance P and CGRP, which are nowadays regarded as the main agents released immediately subsequent to neuronal TRPA1 activation (Nassini et al., 2014).

The mechanism by which TRPA1 enhances inflammation is usually described as neurogenic inflammation. Basically, this cascade begins with an initial stimulus, which directly or via endogenous formation of substances, such as ROS or RNS, activates TRPA1 on the peripheral sensory nerve terminal. The activation of TRPA1 causes a nerve impulse to transmit the noxious sensation but also triggers the release of proinflammatory neuropeptides, particularly substance P, CGRP and neurokinin A, from the activated sensory nerve terminal to the site of the inflammation. The release of these proinflammatory factors drives forward the inflammatory process and may also contribute to a feed-forward mechanism. Furthermore, the afferent impulse towards the central nervous system may propagate antidromically, i.e. opposite to the typical direction of the impulses, down another branch of the same sensory nerve and induce the release of proinflammatory neuropeptides at a distinct but perhaps at a close location to the original onset of stimuli. In addition, after the original afferent impulse reaches the dorsal horn, it may induce a dorsal root reflex in which the dorsal horn interneuronal circuits induce downward impulses towards the periphery in an antidromic manner in the afferent sensory nerves and/or via distinct efferent nerves. An illustrated schematic is presented in Figure 2. (Koivisto et al., 2014; Richardson & Vasko, 2002; Schmelz & Petersen, 2001)

In the year 2009 when we began our studies on TRPA1, the evidence for a proinflammatory role of TRPA1 was only starting to appear. Most of the studies had focused on the proinflammatory properties of direct TRPA1 activators and during the past 7 years it has been extremely interesting to follow the accumulation of reports describing the proinflammatory role of TRPA1. The early results proposed that TRPA1 stimulation via application of direct activators or agents capable of producing endogenous TRPA1 activators could induce the pain, hyperalgesia and allodynia associated with acute inflammation including edema and extravasation (Bandell et al., 2004; Bautista et al., 2005; Matta et al., 2008; Obata et al., 2005; Petrus et al., 2007; Trevisani et al., 2007). However, the role of TRPA1 in many

experimental inflammatory disease models as well as its contribution to the production of proinflammatory factors was still unknown. Nowadays, it has become evident that activation of TRPA1 is involved in the development of experimental inflammatory disease models such as colitis, allergic asthma, atopic dermatitis and gout (Caceres et al., 2009; Engel et al., 2011; B. Liu et al., 2013; Trevisan, Materazzi et al., 2013) and our studies have also largely contributed to this new perspective. The underlying details and associated mechanisms related to TRPA1 in inflammation will be evaluated further in the Discussion section.

1.3 Inflammation

Inflammation is a protective host defence mechanism triggered by various factors such as invading microbes or damaged tissue. Inflammation ultimately aims at the removal of the foreign invaders or the repair of the damaged tissue and the consequences of the disruption in the tissue and the restoration of host homeostasis. Even though, in principle, inflammation is beneficial and in many cases vital to the host, if inappropriately directed or controlled, then inflammation may be harmful. Once the acute inflammation is triggered, it may proceed to a chronic phase in which the inflammation is prolonged and differently orchestrated. Normally inflammation is self-limiting and followed by a repair process. (Kumar et al., 2010)

In acute inflammation, a complex and variable cascade of events is inititated within a few minutes and this acute phase may last up to several days. The major components of the acute inflammation are vasodilation, an increase in capillary permeability and infiltration of leukocytes to the site of inflammation. These events are regulated and driven by various proinflammatory mediators which are produced first in response to the initial stimuli and thereafter by the actions of a variety of cell types and processes. All inflammatory mediators have their own molecular targets and functions; i.e. some have vasoactive properties, some are involved in the recruitment of leukocytes, others act to combat the invading pathogens and some evoke nociception. Together they form the "inflammatory soup" meaning the environment of soluble factors associated with acute inflammation. The list of these factors is long, but the role of cytokines such as TNF-α, IL-1 and IL-6, bradykinin, complement components, histamine and serotonin, chemokines, ROS and RNS, neuropeptides substance P, CGRP and neurokinin A and various prostaglandins such as PGE_2 are frequently highlighted. Furthermore, vasodilation leads to increased blood flow and elevated capillary permeability to allow infiltration of fluid

and proteins and to induce movement of inflammatory cells to the site of inflammation. The inflammatory cells, such as the neutrophils, are attracted by various chemotactic agents and once at the inflammatory site, they act together with the resident cells to regulate the inflammatory cascade. Local symptoms of acute inflammation include edema, redness, heat, pain and loss of function. In addition, acute inflammation may exert systemic effects such as alterations in the blood pressure and cardiac output or fever. (Kumar et al., 2010; Rang et al., 2015b)

Once the initial stimuli of the acute inflammation and its consequences are resolved, the repair processes aim at replacing the damaged tissue, either by regeneration of the original tissue or via the formation of fibrous tissue i.e. scarring. Both of these repair mechanisms are frequently involved and may also happen consecutively. Sometimes the tissue may not heal fully and despite the repair attempts, it will remain not fully functional. (Kumar et al., 2010)

Acute inflammation may evolve into a chronic state if the original cause of the inflammation cannot be resolved or the regulatory mechanisms fail to keep the inflammatory reaction in check, but inflammation may have chronic features from its very onset. Chronic inflammation obviously acts over a longer time frame than acute inflammation, ranging from weeks to months or years but it is morphologically also different from its acute counterpart. One characteristic of chronic inflammation is that the infiltrating leukocytes are predominantly mononuclear cells such as macrophages, lymphocytes and plasma cells and the ongoing tissue damage and repair processes lead to fibrosis and angiogenesis. Chronic inflammation is usually subdued in intensity compared to its acute counterpart, but it may include features of acute inflammation or undergo exacerbations. In some cases, the categorization between acute and chronic inflammation is problematic, even unnecessary. Many inflammatory diseases are chronic in their nature such as rheumatoid arthritis, atherosclerosis and chronic obstructive pulmonary disease. (Kumar et al., 2010; Rang et al., 2015b)

This thesis examines the proinflammatory role of TRPA1 largely because of its involvement in neurogenic inflammation. Neurogenic inflammation refers to the neuronal component of the inflammatory reaction, which acts jointly with the classical players involved in inflammation, such as immune cells and blood vessels. The sensory nervous system contributes to the host defence by innervating the tissues exposed to the external environment; it issues an alert to warn the organism that it has been exposed to some harmful substance. Indeed, peripheral afferent nerve terminals detect many inflammatory substances including cytokines such as

IL-1 and TNF-α, exogenous and endogenous danger signals such as acidity, chemicals, ROS, RNS and adenosine triphosphate and pathogen-associated molecular patterns. These inflammatory signals activate the afferent sensory nerves to release neuropeptides which mediate inflammatory functions such as vasodilation, extravasation, chemotaxis and activation of inflammatory cells. In particular, the vascular effects, sometimes in anatomical terms even being separate from the inflammatory locus, are major components of the neurogenic inflammation. The most notable proinflammatory neuropeptides are substance P and CGRP, but other ones such as, adrenomedullin, neurokinins A and B, vasoactive intestinal peptide, neuropeptide Y and gastrin releasing peptide have been identified. In addition to the local effects, the nervous system also contributes to the systemic responses in inflammation that constitute the regulation of blood pressure, bronchial tone and the extent and direction of blood flow between different organs. (Chiu et al., 2012)

1.4 Carrageenan-Induced Acute Paw Inflammation

Carrageenan-induced acute paw inflammation is an experimental animal model widely used to study acute inflammation and also exploited as a screening tool in anti-inflammatory drug research. Injection of carrageenan into the paw of the experimental animal (usually a rodent) launches an acute inflammation and it is usually evaluated via the development of paw edema (Henriques et al., 1987; Morris, 2003). Carrageenan-induced acute paw inflammation follows the pattern of classical acute inflammatory reaction. The predominant cell types initially involved are neutrophils and macrophages and classical inflammatory mediators such as histamine, serotonin, bradykinin, NO, prostanoids, TNF-α, myeloperoxidase (MPO), and IL-6; these all contribute to the development of the inflammatory response (Bolam et al., 1974; Henriques et al., 1987; Nantel et al., 1999; Portanova et al., 1996; Posadas et al., 2004; Rocha et al., 2006; Wirth et al., 1991). The reaction induced by carrageenan is initiated by a rapid release and/or production of bioactive agents such as bradykinin, histamine, ROS and RNS and this reaction is within hours followed by an infiltration of blood derived polymorphonuclear cells (Morris, 2003). Interestingly, the carrageenan-induced inflammatory reaction is biphasic with the first phase of edema peaking at 4 to 6 hours after the injection and being followed by a second peak at 72 hours accompanied by systemic effects, such as an increase in the levels of circulating leukocytes (Henriques et al., 1987). Injection of carrageenan into the paw of the studied rodent also elicits activity in the nociceptors which can be recorded electrophysiologically and also as allodynia and hyperalgesia,

further leading to an increase of sensory nerve innervation (Chakrabarty et al., 2011; Fletcher et al., 1996; Pertovaara et al., 1998; Tabo et al., 1998).

1.5 Gout

Gout is an inflammatory arthritis affecting typically the metatarsal-phalangeal joint of the big toe. Name gout descends from the Latin word *gutta* meaning 'drop' which refers to the ancient theory of the etiology of the gout: the balance of the body humors was believed to be disrupted and an excess of one of the humors would drop into the gout affected joint. The fiery and paroxysmal arthritis was described already in 2640 BC by Egyptians and the association to upper socioeconomic class with its hedonistic lifestyle was well noted. Hence, the gout has also been called 'the king of diseases and the disease of kings', e.g. King Henry VIII of England (1491 – 1547) and King George IV of the United Kingdom (1762 – 1830) have both been proposed to have suffered from gout (Andriote, 2012; Bywaters, 1962). The association to crystal accumulation within the joint as a possible cause to gout was made in the late 17th century and the identification of hyperuricemia as a source of urate crystals led the way to the Nobel Prize winning invention of allopurinol in 1963 as a urate lowering drug to treat gout (Elion, 1989). Nowadays, the acute inflammation is emphasized in the pathology of gout. (Nuki & Simkin, 2006; Shi et al., 2010)

The prevalence of gout is 1-2% in modern world depending on the studied population. The main risk factors associated with gout include age, male gender, purine-rich foods (meat and seafood), high alcohol intake, obesity, renal transplantation and cyclosporine treatment (Saag & Choi, 2006). The disease is characterized by acute arthritis, i.e. gout flare, including symptoms such as local intense pain and hyperalgesia, edema, heat and sometimes systemic symptoms such as fever. As a consequence of a purine metabolism disorder leading to hyperuricemia, the accumulation of monosodium urate (MSU) crystals into the affected joint is largely believed to be the cause of the disease. The non-soluble MSU crystals induce several inflammatory mechanisms in the leukocytes. Firstly, binding of MSU crystals to various cell membrane receptors, such as the Toll-like receptors, leads to NF-κB activation and consequently to enhanced expression of many proinflammatory genes, especially that of IL-1β. MSU crystals are also intensively phagocytosed by the local inflammatory cells which activates intracellular cascades such as spleen tyrosine kinase, phosphoinositide 3-kinase and production of ROS. Next, these intracellular

signals contribute to the activation of the NALP3 inflammasome which results in caspase-1 mediated cleavage of pro-IL-1β into its active form, further enhancing the inflammatory reaction (Kingsbury et al., 2011; Punzi et al., 2012; Shi et al., 2010).

1.6 Monosodium Urate-Induced Experimental Gout

Gout may be studied by the application of MSU crystals to the studied cells or experimental animals. The most widely used experimental animals have been rat and mouse and MSU crystals have been mainly injected into the subcutaneous tissue in the paw, intra-articularly to the knee or ankle joint or into an artificial air-pouch which resembles the synovial joint. An injection of MSU crystals into the paw of the rodent induces an acute inflammatory edema within hours after the injection (Denko & Whitehouse, 1976). MSU crystal-induced acute paw inflammation is susceptible to treatment with classical anti-inflammatory drugs such as the non-steroidal anti-inflammatory drug, indomethacin and the glucocorticoid, prednisone (Denko & Whitehouse, 1976; Fitzgerald et al., 1971). If the MSU crystals are injected into the knee or the ankle, a painful arthritis develops within a day, and the nociceptive behaviour can be measured by analyzing the weight distribution between the affected and non-affected limb or by testing hypersensitivity to a cold or mechanical stimulus (Coderre & Wall, 1987; Otsuki et al., 1986). The nociceptive behaviour can be alleviated by treatment with various anti-inflammatory and analgesic drugs such as indomethacin, ketoprofen and morphine and also by targeting IL-1β with a soluble decoy receptor (Otsuki et al., 1990; Torres et al., 2009).

Since the rodents' joints are extremely small and it is very challenging to obtain reliable synovial fluid samples, an alternative method for mimicking the synovial joint cavity has been developed. A subcutaneous air-pouch can be created by injecting sterile air under the skin of the studied animal; it is known that an internal cell lining resembling a synovial membrane forms into the air-pouch within approximately a week (Forrest, 1988). The subcutaneous air-pouch may be used to obtain an experimental gouty inflammation by injecting MSU crystals into the air-pouch. Extravasation, an accumulation of inflammatory cells (mainly neutrophils) and the production of many classical proinflammatory factors associated with acute inflammation such as IL-1β, IL-6, macrophage inflammatory protein-1α (MIP-1α) TNF-α, PGE_2 and leukotriene B_4 have been determined in studies evaluating the MSU crystal-induced air-pouch inflammation (Brooks et al., 1987; Inokuchi et al., 2008; Jung et al., 2007). Furthermore, the non-steroidal anti-inflammatory drugs,

such as indomethacin and rofecoxib, and anti-inflammatory glucocorticoids, including prednisolone and betamethasone, have been found to be effective in attenuating the MSU crystal-induced air-pouch inflammation (Forrest et al., 1988; Nalbant et al., 2005; Rull et al., 2003).

1.7 Osteoarthritis

Osteoarthritis is the most common joint disease worldwide and it affects every tenth man and every fifth woman over 60 years of age (Woolf & Pfleger, 2003; Y. Zhang & Jordan, 2010). Osteoarthritis is a significant source of individual suffering due to pain and disability but it is also a major socioeconomic burden to communities. There are several recognized risk factors for osteoarthritis, e.g. age, female sex, obesity, joint traumas, occupational factors, low physical activity and genetic factors (Musumeci et al., 2015). The affected joints are often weight bearing large synovial joints, i.e. hip and knee joint, but the disease may occur also in smaller joints such as the interphalangeal joints of the hand. Osteoarthritis is considered to be a degenerative joint disease driven by a low-grade inflammation and it leads to destruction of articular cartilage causing symptoms such as pain and disability (Figure 3). It is known that many cell types like synoviocytes, osteoblasts and especially chondrocytes exhibit inflammatory activity and alter the cartilage turnover to a negative balance. The inflammatory reaction includes many well-known proinflammatory cytokines such as IL-1β, IL-6 and TNF-α and their role in increasing the local production of collagenases and aggrecan-degrading enzymes has been highlighted in the pathology of osteoarthritis. However, there is still no full and integrated understanding of the development of osteoarthritis. At present, the treatment of osteoarthritis is based on analgesic drugs, physical exercise, reduction of overweight and ultimately joint replacement surgery. Despite intense efforts, osteoarthritis still lacks disease modifying treatment and therefore further investigations are needed. (Glyn-Jones et al., 2015)

Based on previous knowledge, it is possible that TRPA1 has some relevance in osteoarthritis. The proinflammatory neuropeptide substance P has been implicated in the inflammatory reaction triggered in chondrocytes (O'Shaughnessy et al., 2006). Furthermore, there is some direct evidence for the expression of substance P receptor, i.e. neurokinin-1 (NK1) receptor in chondrocytes (Opolka et al., 2012). A proinflammatory role of ROS has also been postulated in osteoarthritis since

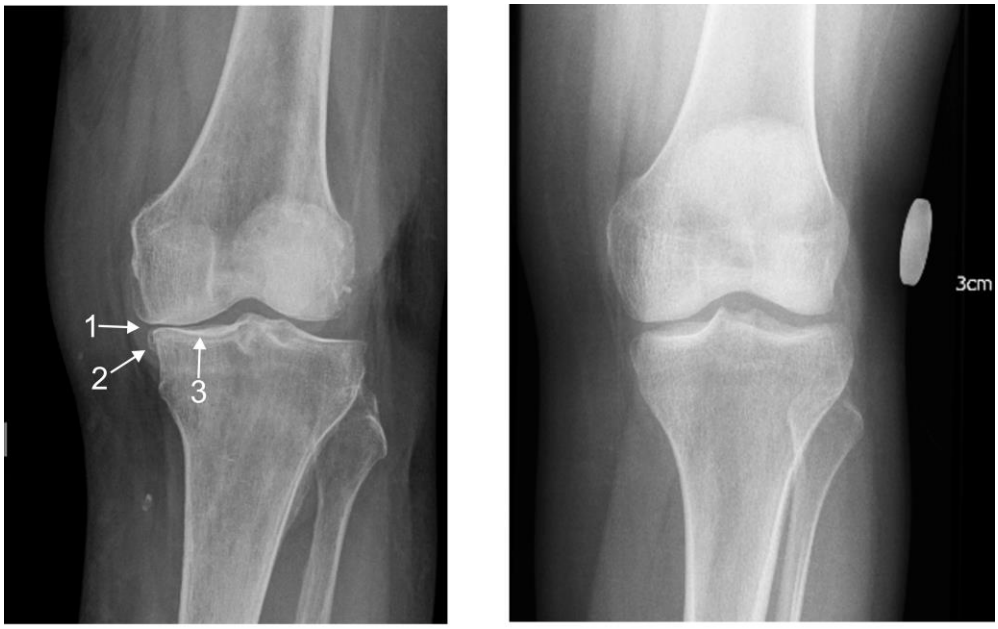


Figure 3. Radiographic image of osteoarthritic and normal left knee joint. The left image reveals the significant osteoarthritic changes characteristic for cartilage and joint degeneration. Narrowing of joint space (1), formation of osteophytes (2) and sclerosis of subchondral bone (3) are typical radiographic findings of osteoarthritis. The image on the right represents a normal knee joint radiography.

inhibiting the action of ROS has attenuated the production of cartilage degrading matrix metalloproteinases (Reed et al., 2014). At this point, it should be noted that in large clinical trials, oral antioxidant therapy has mainly failed to cure or even relieve inflammatory diseases (Firuzi et al., 2011). Therefore, combating ROS in inflammatory diseases should be addressed as a part of the inflammatory reaction as whole and a more direct and possibly more comprehensive approach to inhibiting the action of ROS locally could be beneficial.

1.8 Monosodium Iodoacetate-Induced Experimental Osteoarthritis and Inflammation

An injection of monosodium iodoacetate (MIA) into the articular cavity of an experimental animal, most frequently a rat or a mouse, induces an experimental

osteoarthritis (Marker & Pomonis, 2012; van der Kraan et al., 1989). A few weeks after the application of MIA, histological changes in the cartilage, synovium and subchondral bone and pain-like behaviour in the studied animal can be observed (Bove et al., 2003; Fernihough et al., 2004; van der Kraan et al., 1989). It has been determined that MIA disrupts the cell metabolism in chondrocytes by inhibiting the glyceraldehyde-3-phosphate dehydrogenase, leading to caspase activation and cell death (Dunham et al., 1992; Grossin et al., 2006; Jiang et al., 2013). Simultaneously, inflammation is also induced. *In vitro* studies have detected an increase in the production of many proinflammatory factors such as matrix metalloproteinase-13, IL-1β, IL-6, IL-15, inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) (Dumond et al., 2004; J. Lee et al., 2013). Interestingly, oxidative stress is also involved since there is increased production of ROS (Jiang et al., 2013). The nociceptive behaviour is seen in the studied animals as an increase of hyperalgesia and allodynia and a spontaneous decrease in weight borne by the affected limb (Marker & Pomonis, 2012). Ongoing pain can be demonstrated by the fact that the spontaneous firing of nociceptive C-fibers and mechanically induced firing of mechanosensitive A-fibers are increased (Kelly et al., 2012). Furthermore, the development of MIA-induced experimental osteoarthritis has been proposed to lead to central neuronal sensitization. In addition, the dorsal root ganglia of the innervating neurons exhibit an increase in the levels and activity of neuronal injury markers and signalling molecules mediating the neuropathic pain such as p38 phosphorylation (Ferreira-Gomes et al., 2012; Kelly et al., 2012; Y. Lee et al., 2011). Hence, most likely the MIA-induced experimental osteoarthritis involves an inital inflammatory phase followed by the development of cartilage degradation and a subsequent activation of the neuronal mechanisms contributing to the pain evident in the behavioural and electrophysiological recordings.

Previously, two studies have been conducted on the role of TRPA1 in the MIA-induced experimental arthritis, both focusing on the aspect of pain. The first study reported that a single systemic dose of a TRPA1 blocker was effective in attenuating mechanically induced firing in the wide dynamic range neurons, i.e. sensory neurons susceptible to a variety of stimuli, when measured 21 days after the MIA injection (McGaraughty et al., 2010). In the second study, a single dose of TRPA1 blocker, administered either systemically or intra-articularly, failed to attenuate the nociceptive behaviour measured in a weight-bearing test immediately or several days after the injection of MIA into the joint (Okun et al., 2012). However, the studies did not focus on the role of TRPA1 in the development of the MIA-induced inflammation and cartilage changes, leaving unanswered the question of whether

TRPA1 could play a role in the initial progress of the MIA-induced experimental osteoarthritis by driving the inflammatory phase onwards.

1.9 Allergic Inflammation

Allergic inflammation is the basis for a large and diverse group of atopic diseases, such as allergic asthma, allergic rhinitis and a part of atopic eczema, which together have a high prevalence, about 5-20% in worldwide terms (Miescher & Vogel, 2002). The underlying mechanisms leading to allergic inflammation begin with the process of immunization towards the specific allergen. When the immune system initially encounters the allergen, it is phagocytosed by tissue resident antigen presenting cells such as epithelial dendritic cells and then presented to the naïve CD4+ T cells. The T cells differentiate into T helper 2 (T_H2) cells which in turn interact with B lymphocytes; these cells undergo a class switch into the immunoglobulin E (IgE) producing isotype and start to produce circulating IgE specific to the allergen. Circulating IgE binds to FcεRI receptors on the surface of the mast cell and the mast cells become susceptible to activation by the specific allergen. (Abbas et al., 2014)

Allergic inflammation begins during the body's exposure to a previously encountered allergen as the allergen binds to its specific IgE and FcεRI receptor complex on the tissue resident mast cell's plasma membrane triggering mast cell activation. Activation of the mast cells leads to their rapid degranulation and release of factors such as histamine, serotonin, ROS and RNS, resulting in the early-phase response of the allergic reaction marked by vascular effects causing edema and itch. Subsequently, additional proinflammatory factors are secreted including lipid mediators such as leukotrienes and prostaglandins, which also exert vascular effects. Mast cells also produce many cytokines such IL-4, IL-5 and IL-13 which promote the late-phase of allergic inflammation and contribute to the recruitment of circulating leukocytes. Other inflammatory cells, especially eosinophils and T_H2 cells, migrate into the site of inflammation and further enhance the late-phase allergic inflammation. (Abbas et al., 2014)

The classical allergic type 1 hypersensitivity reaction is supplemented by other complementary mechanisms which also contribute to the allergic inflammation. Neurogenic mechanisms have been studied in the development of allergic inflammation and there is evidence of a two-way regulatory interaction between innervating sensory nerves and local immune cells such as eosinophils and mast cells (Cevikbas et al., 2007). The release of the proinflammatory neuropeptide, substance

P, displays a proinflammatory role in models of experimental allergen-induced asthma, conjunctivitis and dermatitis (Ramalho et al., 2013; Scholzen et al., 2001; Yamaji et al., 1997). Furthermore, substance P seems to enhance inflammatory activity in many of the immune cells known to be involved in allergic inflammation including mast cells, eosinophils and keratinocytes (Asadi et al., 2012; Paus et al., 1995; Raap et al., 2015). There is also convincing evidence that substance P mediates the symptoms of allergic inflammation as treatment with the blocker of the substance P receptor, i.e. the neurokinin-1 receptor, is effective in alleviating itch both in animal studies and in humans (Costantini et al., 2015; Stander et al., 2010).

TRPA1 activation has also been associated with allergic inflammation. The first breakthrough in this field was the discovery of the proinflammatory role of TRPA1 in ovalbumin-induced experimental murine asthma (Caceres et al., 2009). Blockade of TRPA1 by pharmacological treatment or genetic depletion of TRPA1 achieved an attenuation of both airway hyperreactivity and a reduction in the release of proinflammatory factors including IL-5, IL-13, TNF-α, CGRP, neurokinin A and substance P (Caceres et al., 2009). It is noteworthy that the immunization towards the allergen ovalbumin was undisturbed.

Experimental allergic contact dermatitis, resembling the type IV or delayed type hypersensitivity, can be induced by the application of oxalozone. Intriguingly, oxalozone has been proven to be a direct activator of TRPA1 and in this model TRPA1 deficient mice exhibited diminished levels of leukocyte accumulation, less plasma extravasation and decreases in the amounts of proinflammatory factors such as IL-4, histamine and substance P when measured from the inflamed tissue and these animals also exhibited attenuated itch behaviour (B. Liu et al., 2013). Hence, it is possible that TRPA1 activation plays a role also in the delayed type hypersensitivity and could possibly contribute to the different mechanisms involved in the different types of hypersensitivity reactions and allergic inflammation.

Another study presented parallel results as also atopic dermatitis induced by IL-13 was reported to be TRPA1-dependent and intriguingly TRPA1 expression was elevated not only in innervating sensory nerves but also in resident mast cells (Oh et al., 2013). In addition, leukotriene B_4, a factor also associated with allergic inflammation, has been shown to increase TRPA1 expression in mast cells (Fernandes et al., 2013). The association between TRPA1 and mast cells is also evident in the TRPA1 activation-induced edema. A depletion of mast cell degranulation or inhibition of the main products of mast cell degranulation, i.e. serotonin and histamine, leads to diminished *in vivo* edema formation seen after the

application of the TRPA1 activator AITC even though AITC does not directly induce mast cell degranulation (Perin-Martins et al., 2013; Trevisan, Rossato et al., 2013). Although the role of TRPA1 in allergic inflammation has been studied from fairly broad perspectives, the results have not identified the exact mechanisms or effector cell type involved to explain how TRPA1 contributes to the allergic inflammation and therefore further studies are needed.

1.10 Ovalbumin-Induced Experimental Allergic Inflammation

Ovalbumin is a 45 kDa glycoprotein constituting up to 55% of the protein in egg white and it is one of the major allergens causing food allergy towards eggs in humans (Caubet et al., 2011). Although a target of extensive research, the physiological function of ovalbumin in the chicken embryo is unknown although it is assumed to act as a storage protein.

Ovalbumin is frequently used as an allergen in experimental allergy research. The experimental animals, usually rodents, are immunized towards ovalbumin via an exposure to ovalbumin frequently combined with adjuvants such as $Al(OH)_3$ and bacterial toxins and hence the animals form specific IgE towards the allergen (Chung et al., 2012; Reddy et al., 2012). Next, the studied animals can be challenged with ovalbumin by various routes to induce an experimental allergic inflammation. In addition to airway exposure leading to bronchial hypersensitivity, the application of ovalbumin may be carried out topically to the eye to produce an allergic conjunctivitis (Reddy et al., 2012). The topical ocular challenge induces the release of several proinflammatory mediators such as histamine and NO and also results in infiltration of leukocytes, especially eosinophils, into the inflamed conjunctival tissue (Chung et al., 2012; Helleboid et al., 1991; Meijer et al., 1996). The conjunctivitis induces also physical symptoms such as hyperemia and increased lacrimation (Hayat et al., 2011). Ovalbumin-induced allergic experimental conjunctivitis can be inhibited by treatment with substances such as the glucocorticoid dexamethasone the immunosuppressant cyclosporine A and with an NO scavenger (Meijer et al., 1996; Shii et al., 2010; Shoji et al., 2005). Interestingly, a similar allergic inflammation resembling the classical type I hypersensitivity can be induced by injecting ovalbumin into the paw of a previously immunized animal. Although not so often used, this model of ovalbumin-induced allergic paw inflammation evokes an acute paw edema which can be inhibited by treatment with serotonin receptor inhibitors and the glucocorticoid dexamethasone (Feitosa et al., 2002).

1.11 Pinosylvin

Pinosylvin (3,5-dihydroxy-trans-stilbene) is a stilbenoid polyphenol; it is found in natural sources such as in the heartwood and bark of the coniferous tree species *Pinus*. Pinosylvin has been shown to possess anti-inflammatory and cancer chemopreventive properties in addition to its chemical functions as an antioxidant preventing oxidative stress (Koskela et al., 2014; Laavola et al., 2015; Macickova et al., 2010; Park et al., 2012). Pinosylvin displays some structural similiarity to a more extensively studied stilbenoid, resveratrol, which has been described to have antioxidant, anti-inflammatory, antiproliferative and chemoprotective properties and many molecular targets (Kulkarni & Canto, 2015). Intriguingly, resveratrol has been identified as a blocker of TRPA1 (Yu et al., 2013) and therefore stilbenoids such as pinosylvin may represent a treasure-trove of novel drugs capable of inhibiting TRPA1.

2 Aims of the Study

During the past ten years, research on TRPA1 ion channel has revealed it to be a potent chemosensor and nociceptor. In addition to exogenic irritating compounds, also reactive molecules formed in the inflammatory reaction have been demonstrated to act as TRPA1 activators. Interestingly, the activation of TRPA1 has been shown to be involved not only in acute pain and hypersensitivity but also in neurogenic inflammation. TRPA1 blockers are under intense research and development but the exact indications for TRPA1 blocking drug treatment are still to be discovered. Hence, the aim of the study was to assess the possible proinflammatory role of TRPA1 by utilising experimental models of inflammatory diseases with different pathogenetic mechanisms.

The detailed aims of this study were:

1. to study the possible role of TRPA1 in acute inflammation in carrageenan-induced inflammatory paw edema and the association between TRPA1 activation and the prostaglandin producing COX-enzyme (study I)

2. to evaluate the putative role of TRPA1 in experimental gouty inflammation by measuring acute inflammation and joint pain (study II)

3. to assess the contribution of TRPA1 activation in MIA-induced experimental osteoarthritis by evaluating MIA-induced acute inflammation and the development of cartilage changes and joint pain (study III)

4. to clarify the potential involvement of TRPA1 activation in experimental ovalbumin-induced acute allergic inflammation (study IV)

5. to investigate the possible effects of pinosylvin on TRPA1-mediated responses *in vitro* and *in vivo* by measuring AITC-induced Ca^{2+} influx and paw edema, respectively (study V)

3 Materials and Methods

3.1 Compounds

All reagents were purchased from Sigma Chemical Co., St. Louis, MO, USA unless otherwise indicated. The pharmacological tools used are listed in Table 5.

The MSU crystals used in the study II were prepared as previously described (Denko & Whitehouse, 1976) by diluting 1.0 g of uric acid in 200 ml of water adjusted to pH 14.0 with NaOH by heating and blending. Next, the pH was gradually adjusted to 7.0 by adding HCl and MSU was crystallized overnight at room temperature with constant shaking. The formed crystals were filtered, washed, dried and re-suspended in phosphate buffered saline (PBS) at a concentration of 50 mg/ml. In the microscopic examination, the MSU crystals were 5-20 μm in length (Figure 4). All equipment and liquids used were endotoxin free.

3.2 Mice

In the first study (I), the wild type (WT) and TRPA1 deficient (or knock-out, KO) C57BL/6 mice were originally obtained from Dr David Julius (UCSF) and back-crossed in the laboratory of Edward Högestätt and Peter Zygmunt (Lund University, Sweden). In the other studies (II-IV), the wild type and TRPA1 deficient B6;129P-Trpa1(tm1Kykw)/J mice from Charles River Laboratories, Sulzfeld, Germany were used in the experiments. The genotypes of the mice were confirmed by polymerase chain reaction (PCR). The TRPA1 deficient mouse strains used are further described in the Discussion section in paragraph 5.1.1 and in the original publications (Bautista et al., 2006; Kwan et al., 2006). In the third, fourth and fifth study (III-V), the drug effects were studied by using wild type C57BL/6 mice (Scanbur Research A/S, Karlslunde, Denmark). Mice were housed under standard conditions (12-12 h light-dark cycle, 22±1 °C) with food and water provided *ad libitum*. All 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.

Table 5. Used pharmacological compounds and their targets

Compound	Target
Allyl isothiocyanate (AITC)	TRPA1 opener
Catalase	H_2O_2 degrading enzyme
Dexamethasone	Anti-inflammatory glucocorticoid
HC-030031; (2-(1,3-Dimethyl-2,6-dioxo-1,2,3,6-tetrahydro-7H-purin-7-yl)-N-(4-isopropylphenyl)acetamide)	Selective TRPA1 blocker
Ibuprofen	COX inhibitor; non-steroidal anti-inflammatory drug
L703,606; (cis-2-(Diphenylmethyl)-N-[(2-iodophenyl)methyl]-1-azabicyclo[2.2.2]octan-3-amine oxalate salt)	NK1 receptor antagonist
Pinosylvin	Stilbenoid, putative TRPA1 blocker
Resveratrol	Stilbenoid, TRPA1 blocker
TCS 5861528; (2-(1,3-Dimethyl-2,6-dioxo-1,2,3,6-tetrahydro-7H-purin-7-yl)-N-[4-(1-methylpropyl)phenyl]acetamide)	Selective TRPA1 blocker

Intraperitoneal injection 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 for anesthesia. Animals were sacrificed after experiments by carbon monoxide followed by cranial dislocation.

To study drug effects, the mice were dosed with HC-030031 (300 mg/kg perorally or intraperitoneally), ibuprofen (100 mg/kg intraperitoneally), dexamethasone (2 mg/kg perorally or intraperitoneally), TCS 5861528 (10 mg/kg perorally), catalase (300 IU/paw by intraplantar administration) L703,606 (10 mg/kg intraperitoneally, Enzo Life Sciences AG, Lausen, Switzerland), pinosylvin (10 mg/kg intraperitoneally) or resveratrol (10 mg/kg intraperitoneally). Orally administered drugs were diluted in 75% polyethylene glycol and given by gastric 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 MIA (study III) or ovalbumin (study IV).

3.3 Acute Paw Edema

Acute paw edema was induced by injecting carrageenan (450 µg, study I), AITC (20 µg, study I; 50 µg, study V) MSU crystals (500 µg, study II), MIA (400 µg, study III) or ovalbumin (45 µg, study IV) diluted in sterile endotoxin free PBS or NaCl 0.9% (30 µl, study I; 50 µl, studies II-V). The contralateral paw was injected with the vehicle and developed neglible edema. The paw volume was measured up to 6 h with a plethysmometer (Ugo Basile, Comerio, Italy) and compared to the baseline value. Mice given drug treatments were compared to vehicle treated mice and TRPA1 deficient mice were compared to the corresponding wild type mice.

In some acute paw edema experiments (studies III-V), after the mice had been sacrificed, the inflamed subcutaneous paw tissue injected with the stimuli and the contralateral subcutaneous paw tissue injected with the vehicle were collected for analysis in 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 firm shaking. The samples were centrifuged (10 min, 10 000 *g*) and the supernatant was collected and measured for substance P (study III), IL-4 (study IV) or IL-6 (study V) with an enzyme-linked immunosorbent assay (ELISA; R&D Systems Europe Ltd., Abingdon, UK).

3.4 Monosodium Urate-Induced Acute Arthritis

The MSU crystal-induced weight-bearing test originally described in 1986 (Coderre & Wall, 1987; Otsuki et al., 1986) was triggered by injecting 500 µg of MSU crystals in 40 µl of sterile endotoxin free PBS into the knee joint of anesthetized mice. The contralateral knee joint was injected with the corresponding volume of the vehicle.

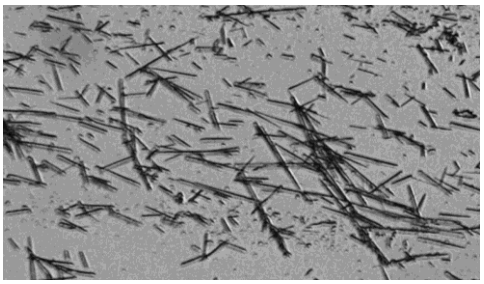


Figure 4. Light microscopic image of MSU crystals. The MSU crystals were prepared as described in the Materials and Methods section and photographed during the light microscopic assessment with 100 x magnification. The crystals are razor-sharp in shape and 5-20 µm in length. Photograph by Mari Hämäläinen.

The willingness to bear weight on the affected joint was measured with an incapacitance meter (IITC Life Science, Woodland Hills, CA, USA) for four subsequent 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 8 times for 1 second at each time point and the investigator was blinded towards the affected limb.

3.5 Monosodium Urate-Induced Air-Pouch Inflammation

A subcutaneous air-pouch was created by injecting 3 ml (1st day) and 1.5 ml (3rd day) of sterile air subcutaneously under the dorsal skin of the studied mice under anaesthesia and after 7 days a synovial-like epithelium has been shown to be present in the air-pouch (Forrest, 1988). The inflammation was induced by injecting 3 mg of MSU crystals in 1 ml of sterile endotoxin-free PBS into the air-pouch of the anesthetized mice. After 6 h, the mice were sacrificed and the exudate was harvested for cell-counting by hemocytometer and for cytokine measurements. Monocyte chemotactic protein-1 (MCP-1), IL-6, IL-1β, MPO, macrophage inflammatory protein-1α (MIP-1α) and macrophage inflammatory protein-2 (MIP-2) were measured by ELISA (R&D Systems).

3.6 Monosodium Iodoacetate-Induced Osteoarthritis

The MIA-induced weight-bearing test was initiated by injecting MIA (500 µg) diluted in 40 µl of sterile endotoxin free PBS into a randomized knee joint of anesthetized mice. The contralateral knee joint was injected with the corresponding volume of the vehicle. The willingness to bear weight on the affected joint was measured with

an incapacitance meter (IITC Life Science) 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 8 times for 1s at each time point and the investigator was blinded towards the affected limb.

After the weight-bearing test at 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 for 48 h in Osteomoll (Merck, Darmstadt, Germany) which contains HCl (10%) and CH_2O (4%) and embedded in paraffin. Then 5 µm thick coronal sections 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 (Glasson et al., 2010) by two independent observers who were blinded for the treatment and genotype.

3.7 Ovalbumin-Induced Conjunctivitis

The studied mice were immunized to ovalbumin with an intraperitoneal injection of ovalbumin (100 µg), $Al(OH)_3$ (1 mg, Acros Organics, Geel, Belgium) and Pertussis toxin (300 ng) suspended in 550 µl of sterile PBS on day 1. Immunization was boosted with an intraperitoneal injection of ovalbumin (100 µg) and $Al(OH)_3$ (1 mg) suspended in 500 µl of sterile PBS on day 5. Non-immunized control mice received intraperitoneal injections of PBS only. The experiments were performed on day 14 after the immunization.

Ovalbumin-induced allergic conjunctivitis was triggered by applying topically first DL-dithiothreitol (770 µg suspended in 5 µl of sterile PBS) and 15 min later ovalbumin (500 µg suspended in 5 µl of sterile PBS) into both eyes of the studied mice earlier immunized to ovalbumin. Both eyes of the studied mice were treated topically by applying TRPA1 blocker TCS 5861528 (25 µg suspended in 5 µl of sterile PBS), the control compound dexamethasone (5 µg in 5 µl, Oftan Dexa®, Santen, Tampere, Finland) or vehicle (5 µl of PBS) at 1 h before and 2 h, 4 h and 6 h after the application of ovalbumin. Finally, 24 h after the application of ovalbumin, the mice were sacrificed and conjunctiva from both eyes were surgically removed and analysed for eosinophil infiltration by measuring eosinophil peroxidase activity.

To measure eosinophil peroxidase activity, the tissue samples were first washed with ice-cold PBS and then homogenized mechanically with a disposable pellet pestle cordless motor (Kimble Chase Life Science and Research Products LLC., Rockwood, TN, USA) in Tris-HCl buffer (50 mM, pH 8.0) containing 0.1% Triton-X. Subsequently, the samples were incubated at 4 °C for 2 h and then centrifuged (10 min, 10 000 *g*, 4 °C) and supernatants were collected for further analysis. Samples (50 µl) and eosinophil peroxidase substrate solution (100 µl) containing o-phenylenediamine (2 mM) and 0.1% Triton-X in Tris-HCl buffer (50 mM, pH 8.0) were added to a 96-well microplate. The reaction mixture was incubated in the dark for 30 min at room temperature and the reaction was stopped by adding H_2SO_4 (50 µl, 2 M) into each well. Finally, the eosinophil peroxidase activity corresponding to the amount of eosinophils which had infiltrated into the tissue was measured as absorbance at 490 nm by Victor3 1420 multilabel counter (Perkin Elmer, Waltham, MA, USA).

3.8 Cell and Cartilage Culture

3.8.1 HEK 293 Cells

HEK 293 human embryonic kidney cells (American Type Culture Collection, Manassas, VA, USA) were cultured in Eagle's Minimum Essential Medium (EMEM) supplemented with fetal bovine serum (10%), sodium bicarbonate (150 mg/ml), sodium pyruvate (1 mM), non-essential amino acids (1 mM each) (all from Lonza, Verviers SPRL, Verviers, Belgium), penicillin (100 U/ml), streptomycin (100 µg/ml) and amphotericin B (the last three compounds from Invitrogen, Paisley, UK) at 37 °C in 5% CO_2. The cells were transfected for 24 h using 420 ng/cm^2 human TRPA1 plasmid DNA (pCMV6-XL4 by Origene Rockville, MD, USA) with Lipofectamine 2000 (Invitrogen) according to the manufacturer's directions. Next, the cells were washed and used in the Fura-2-AM (study I) or Fluo-3-AM assays evaluating Ca^{2+} influx (studies II-V) or the cells were treated with HC-030031 (10 µM) or solvent (control) 30 min prior to the activation of TRPA1 by AITC (10 µM) for 6 h to measure the expression of COX-2 (study I).

3.8.2 Human Osteoarthritic Chondrocytes

In study III, the leftover pieces of osteoarthritic cartilage from knee joint replacement surgery were used with full patient consent and approval by the Ethics Committee of Tampere University Hospital, Tampere, Finland. Full-thickness pieces of articular cartilage from femoral condyles and tibial plateaus showing macroscopic features of early osteoarthritis were removed aseptically from subchondral bone with a scalpel and cut into small pieces. The pieces were washed in PBS and the chondrocytes were isolated by enzymatically digesting the cartilage tissue by using a collagenase enzyme blend (1 mg/ml, Liberase Research Grade Medium; Roche, Mannheim, Germany) for 16 h at 37 °C. Isolated chondrocytes were washed and plated on 24-well plates (0.15 x 10^6 cells/ml) in Dulbecco's Modified Eagle's Medium (DMEM) with GIBCO GlutaMAX-I supplemented with penicillin (100 U/ml), streptomycin (100 µg/ml), and amphotericin B (250 ng/ml, all from Invitrogen) containing fetal bovine serum (10%). The chondrocytes were cultured at 37 °C in a humidified 5% CO_2 atmosphere for 6 days during which the culture medium was changed on days 2 and 4. Next, the chondrocytes were exposed to MIA (100 µM), the TRPA1 blocker HC-030031 (100 µM), IL-1β (100 pg/ml, R&D Systems) or to a combination of these compounds. The chondrocytes were incubated with the studied compounds for 6 h and analysed by reverse transcription polymerase chain reaction (RT-PCR) for COX-2 expression.

3.8.3 Mouse Cartilage Culture

In study III, after the mice were sacrificed, full-thickness articular cartilage from the femoral heads was removed and incubated at 37 °C in humified 5% CO_2 atmosphere in DMEM supplemented with penicillin (100 U/ml), streptomycin (100 µg/ml), and amphotericin B (250 ng/ml, all from Invitrogen) containing fetal bovine serum (10%). The cartilage pieces were exposed to MIA (10 µM), IL-1β (100 pg/ml), HC-030031 (100 µM) or to a combination of these for 24 h and the samples were analysed for COX-2 expression by Western Blotting.

3.8.4 Eosinophil Isolation, Culture and Viability Determination

Blood derived eosinophils were obtained from healthy human donors and used in the study IV. The subjects provided written informed consent to the study protocol which had been approved by the Ethics Committee of Tampere University Hospital,

Tampere, Finland. Eosinophils were isolated as previously described (Kankaanranta, Lindsay et al., 2000). Briefly, venous blood from healthy individuals was collected into tubes with acid-citrate-dextrose anticoagulant. White blood cells were obtained by sedimentation with 3% hydroxyethyl starch, layered onto Ficoll, and centrifuged at 500 *g* for 30 min at 18 °C. The mononuclear cell layer was discarded and the pellet containing granulocytes and red blood cells was washed in Hank's Balanced Salt Solution. Contaminating red blood cells were lysed with hypotonic lysis buffer. The remaining granulocytes were washed, counted, and re-suspended in 300 µl of Roswell Park Memorial Institute (RPMI) 1640 culture medium (Lonza) containing fetal bovine serum (2%) and EDTA (5 mM) (RPMI/FBS/EDTA). Eosinophils were purified from neutrophils with the addition of immunomagnetic anti-CD16 antibody-conjugated beads (1 µl of beads per 2×10^6 granulocytes). After the addition of the beads, the cells were incubated at 4 °C for 40 min before resuspension in 6 ml of RPMI/FCS/EDTA. The mixture was loaded onto a separation column positioned within a magnetic field and eluted with 40 ml of RPMI/FBS/EDTA. The $CD16^+$ cells, i.e. neutrophils, were retained by the column, whereas the eluted eosinophils were collected, washed in RPMI 1640, counted, and then re-suspended at 10^6 cells/ml. Eosinophil purity was >99% as assessed by microscopic examination after Kimura staining.

The cells were cultured for 48 h (37 °C, 5% CO_2) in RPMI 1640 medium supplemented with penicillin (100 U/ml), streptomycin (100 µg/ml), and amphotericin B (250 ng/ml, all from Invitrogen) containing fetal bovine serum (10%). Next, the cells were incubated with the TRPA1 blockers TCS 5861528 (10 µM) or HC-030031 (10 µM), the TRPA1 channel opener AITC (10 µM) or with the control compound dexamethasone (1 µM) in addition to IL-5 (0.5 pM), which was used to decrease the apoptosis rate in comparison to the basal state.

Fragmentation of DNA by endonucleases is considered as a specific characteristic of apoptotic cells (Kankaanranta, de Souza et al., 2000). Eosinophil apoptosis was determined by using the relative DNA fragmentation assay in propidium iodide-stained cells and flow cytometry. Briefly, after 40 h, the cells were suspended into 300 µl of hypotonic solution (0.1% sodium citrate, 0.1% Triton-X and 50 µg/ml propidium iodide). The cells were washed with PBS, fixed in ethanol (70%) and incubated at 4 °C for 30 min. After a wash in PBS, the cell pellet was resuspended in a buffer containing propidium iodide (50 µg/ml). The cells were incubated at 4 °C for 1 h protected from light and finally the samples were analysed by flow cytometry (FACScan, Becton Dickinson, San Jose, CA, USA) with

excitation/emission wavelengths of 488/550 nm. Reduced relative DNA content was regarded apoptotic and the eosinophil apoptosis was expressed as percentage of apoptotic cells (number of apoptotic cells/total number of cells x100).

3.8.5 Splenocyte Isolation and Interleukin-13 Measurements

In study IV, the immunized animals were sacrificed and their spleens were surgically removed. The spleens were mechanically broken by pressing them through a 70 μm cell strainer. The cells were washed with PBS and suspended in lysis buffer containing NH_4Cl (0.15 M), $KHCO_3$ (0.1 mM) and EDTA (0.1 mM) to break down the erythrocytes in the suspension. After an incubation of 5 min, the isolated splenocytes were washed with PBS and plated on a 24-well microplate at a density of 1 x 10^6 cells/ml and cultured in RPMI 1640 culture medium supplemented with fetal bovine serum (10%), penicillin (100 U/ml), streptomycin (100 μg/ml), and amphotericin B (250 ng/ml, all from Invitrogen) and β-mercaptoethanol (14 μM). The cells were incubated with T-cell activating CD3 and CD28 antibodies (CD3 antibody 5 μg/ml coated -1 h on the culture plate, CD28 antibody 5 μg/ml diluted in culture medium, both from eBioscience, San Diego, CA, USA) or with ovalbumin (100 μg/ml diluted in culture medium) either with or without dexamethasone (1 μM). After 42 h, the culture medium was harvested and analysed for the IL-13 concentration by ELISA (R&D Systems).

3.9 Protein Extraction and Western Blotting

After the cell culture experiments in study III, the mouse cartilage explants were rapidly washed with ice-cold PBS and minced in cold lysis buffer containing RIPA buffer base (pH 7.4, 1% NP-40, 0.25% sodium deoxycholate, 1 mM EDTA), NaCl (50 mM), 1% Triton-X, phenylmethylsulfonyl fluoride (0.5 mM), sodium orthovanadate (1 mM), leupeptin (20 μg/ml), aprotinin (50 μg/ml), NaF (5 mM), sodium pyrophosphate (2 mM) and n-octyl-β-D-glucopyranoside (10 μM). After repeated shaking and overnight incubation at 4 °C in the lysis buffer, the tissue lysates were centrifuged (13 400 *g*, 4 °C, 10 min) and supernatants were collected and stored in SDS sample buffer at −20 °C. An aliquot of the supernatant was used to determine the protein concentration by the Coomassie blue method. Next, equal aliquots of protein (20 μg) were boiled for 5 min and loaded onto a 10% SDS-polyacrylamide electrophoresis gel and electrophoresed for 4 h at 100 V in a buffer

containing Tris–HCl (95 mM), glycine (960 mM), and SDS (0.5%). After electrophoresis, the proteins were transferred to a Hybond enhanced chemiluminescence nitrocellulose membrane (Amersham Biosciences, Buckinghamshire, UK). After transfer, the membrane was blocked in TBS/T (20 mM Tris–base pH 7.6, 150 mM NaCl, 0.1% Tween-20) containing 5% non-fat milk for 1 h at room temperature and incubated in the blocking solution at 4 °C overnight with the primary antibody for COX-2 (1:500, sc-1745, goat polyclonal immunoglobulin G) and loading control actin (1:2000, sc-1616R, rabbit polyclonal immunoglobulin G). On the next day, the membranes were incubated in the blocking solution for 1 h at room temperature with the respective horse radish peroxidase-conjugated secondary antibodies for COX-2 (1:5000, sc-2020, donkey anti-goat polyclonal immunoglobulin G) and actin (1: 2000, sc-2004, goat anti-rabbit polyclonal immunoglobulin G, all antibodies from Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). Bound antibody was detected using Super Signal® West Pico or Dura chemiluminescence substrate (Pierce, Rockford, IL, USA) and ImageQuant LAS 4000 mini imaging system (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). The quantitation of the chemiluminescent signal was carried out with the use of Image Quant TL software (GE Healthcare Bio-Sciences AB).

3.10 Enzyme Linked Immunosorbent Assay

All ELISA kits were purchased from R&D Systems Europe Ltd. and the measurement protocols were carried out in accordance with the manufacturer's instructions.

3.11 RNA Extraction and Reverse Transcription Polymerase Chain Reaction

After the cell culture experiments in studies I and III, RNA extraction was carried out with the use of GenElute Mammalian Total RNA Miniprep Kit. Total RNA (25 ng) was reverse-transcribed to cDNA using TaqMan Reverse Transcription reagents and random hexamers (Applied Biosystems, Foster City, CA, USA). The cDNA obtained from the reverse transcription reaction (amount corresponding to approximately 1 ng of total RNA) was subjected to PCR using TaqMan Universal

Table 6. The primer and probe sequences and concentrations for the studied human genes in the PCR measurements.

Gene		Sequence	Concentration
COX-2	forward primer	5'-CAACTCTATATTGCTGGA-ACATGGA-3'	300 nM
	reverse primer	5'-TGGAAGCCTGTGATACT-TTCTGTACT-3'	300 nM
	probe	5'-TCCTACCACCAGCAACCC-TGCCA-3'	150 nM
GAPDH	forward primer	5'-AAGGTCGGAGTCAACGG-ATTT-3'	300 nM
	reverse primer	5'-GCAACAATATCCACTTTA-CCAGAGTTAA-3'	300 nM
	probe	5'-CGCCTGGTCACCAGGGCTGC-3'	150 nM

PCR Master Mix and ABI PRISM 7000 Sequence detection system (Applied Biosystems). The primer and probe sequences and concentrations were optimized according to the manufacturer's guidelines in TaqMan Universal PCR Master Mix Protocol part number 4304449 revision C and displayed in the Table 6. TRPA1 expression was measured with the TaqMan Gene Expression Assay (Hs00175798_m1, Applied Biosystems). PCR reaction parameters were as follows: incubation at 50 °C for 2 min, incubation at 95 °C for 10 min, and thereafter 40 cycles of denaturation at 95 °C for 15 s and annealing and extension at 60 °C for 1 min. Relative COX-2 mRNA levels were normalized against housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

3.12 Patch Clamp, Fura-2-AM and Fluo-3-AM Measurements

3.12.1 Patch Clamp Measurements

In study V, the whole-cell patch clamp experiments were performed as described previously (Hatano et al., 2012). The resistance of electrodes was 3–5 MΩ when filled with the pipette solution (110 mM Cs-aspartate, 30 mM CsCl, 1 mM $MgCl_2$, 10 mM HEPES, 10 mM EGTA, 2.2 mM $CaCl_2$, 2 mM Na_2ATP [adjusted to pH 7.2 with CsOH]). Membrane currents and voltage signals were digitized into a computer using an analogue-digital converter (PCI6229, National Instruments Japan). Data acquisition and analysis of whole-cell currents were performed using WinEDRV3.38 developed by Dr John Dempster (University of Strathclyde, UK). The liquid junction potential between the pipette and bath solutions (−10 mV) was corrected. A ramp voltage protocol from −150 mV to +100 mV of 400 ms was applied every 5 s from a holding potential of −50 mV. A HEPES-buffered bathing solution (137 mM NaCl, 5.9 mM KCl, 10 mM CsCl, 1.2 mM $MgCl_2$, 14 mM glucose, 10 mM HEPES [adjusted to pH 7.4 with NaOH]) was used. In the present experiments, extra- and intracellular Ca^{2+} was omitted and adjusted to 0.3 μM in the bathing and pipette solution, respectively, to maintain TRPA1 channel activity. All experiments were performed at 25±1 °C.

3.12.2 Fura-2-AM Measurements

Fluorometric calcium imaging with the Fura-2-acetoxymethyl ester (Fura-2-AM) measurement was used in study I. The HEK 293 cells transfected with human TRPA1 were plated in 96-well black-walled plates (Costar, Cambridge, MA, USA) and loaded with Fura 2-AM (1 μM, Invitrogen), probenecid (2 mM) and pluronic acid (20%, Invitrogen) for 1 h at 37 °C. The cells were then washed with PBS containing 140 mM NaCl, 5 mM KCl, 10 mM glucose, 10 mM HEPES, 2 mM $CaCl_2$ and 1 mM $MgCl_2$, and allowed to equilibrate for 30 min in the dark before the start of the experiments. The intracellular calcium concentration was determined at 25 °C in a Flexstation 3 (Molecular Devices, Sunnyvale, CA, USA). Basal emission ratios with excitation/emission wavelengths of 340/380 nm were measured and changes in dye emission ratio (Δ ratio) determined at various times after compound addition.

3.12.3 Fluo-3-AM Measurements

Cultured cells were loaded with Fluo-3-AM (4 μM, Invitrogen) and 0.08% Pluronic F-127 in Hanks' Balanced Salt Solution containing fetal bovine serum (10%), 2.5 mM probenecid and 25 mM HEPES (pH 7.2) for 30 min at room temperature. The intracellular free Ca^{2+} levels were assessed in a Victor3 1420 multilabel counter (Perkin Elmer) at excitation/emission wavelengths of 485/535 nm (Assay of intracellular free calcium in RAW 264.7 cells loaded with fluo-3, 2003). In the experiments, the cells were first pre-incubated with the TRPA1 blocker HC-030031 (100 μM) (Eid et al., 2008), resveratrol (10 μM, study V) or pinosylvin (0.1-100 μM, study V) or the vehicle for 30 min at 37 °C. Thereafter, MSU crystals (1 mg/ml, study II), MIA (100 μM, study III), ovalbumin (1 mg/ml, study IV) or AITC (50 μM) was added and the measurements were continued for 30 s, after which a robust Ca^{2+} influx was induced by the addition of the control ionophore compound, ionomycin (1 μM).

3.13 Statistics

Results are expressed as mean ± standard error of the mean (SEM, studies I-II, IV-V) or 95% confidence interval (study III). Data were analysed with SPSS version 17.0 for Windows software (SPSS Inc, Chicago, IL, USA) by using Student's *t*-test, mixed between-within subjects ANOVA or one-way ANOVA with Bonferroni's or Dunnett's multiple comparison test.

4 Summary of Results

4.1 Role of TRPA1 in Carrageenan-Induced Acute Inflammation (Study I)

4.1.1 TRPA1 Activation Enhances Carrageenan-Induced Paw Edema

Our initial experiments on the role of TRPA1 in acute inflammation began with the classical carrageenan-induced inflammatory paw edema model. Surprisingly, we observed that the inflammatory edema induced by an injection of carrageenan was clearly attenuated by the treatment with the TRPA1 blocker HC-030031 similar to the used control compound, COX inhibitor ibuprofen (Figure 5A). Furthermore, similar results were obtained in the experiments conducted in TRPA1 deficient mice, which developed a greatly reduced edema when compared to the corresponding wild type mice (Figure 5B).

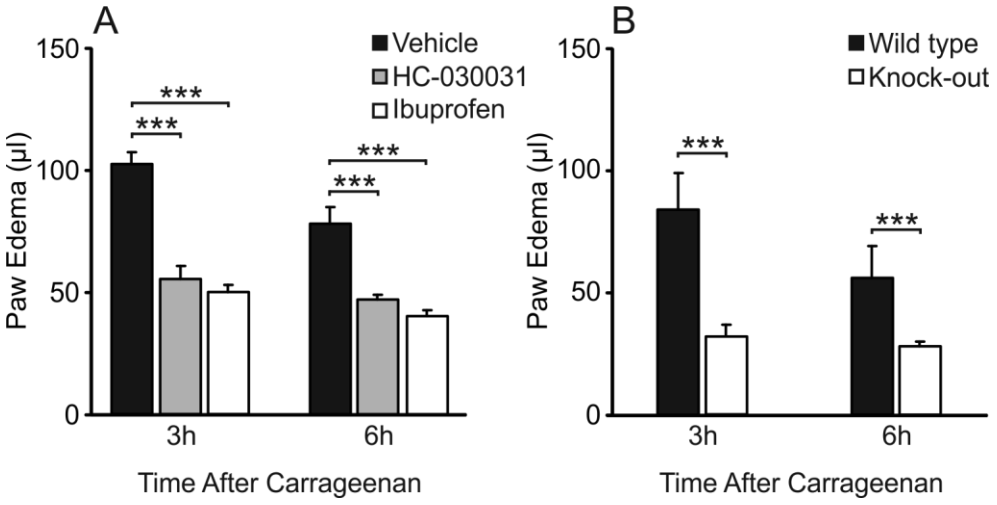


Figure 5. The effect of pharmacological inhibition and genetic depletion of TRPA1 on carrageenan-induced acute inflammatory paw edema. An intraplantar injection of carrageenan (450 µg) induced an acute inflammatory paw edema that was inhibitable by treatment with the TRPA1 blocker HC-030031 (300 mg/kg) or the COX inhibitor ibuprofen

(100 mg/kg) (A). Correspondingly, the carrageenan-induced edema was blunted in TRPA1 knock-out mice as compared to corresponding wild type mice (B). Drugs were given intraperitoneally 2 h prior to the carrageenan injection and the edema was measured with a plethysmometer before and 3 h and 6 h after the carrageenan injection and compared to the basal level. The results are expressed as mean + SEM, n=5-6, ***=p<0.001. Modified from Moilanen et al. Sci Rep, 2012; 2: 380.

4.1.2 TRPA1 Mediates AITC-Induced Edema in a COX-Dependent Manner

An injection of AITC, a well-known TRPA1 opener, triggered an acute paw edema similar to that seen after carrageenan injection. As expected, the evoked edema was inhibited largely by treatment with the TRPA1 blocker HC-030031 and the TRPA1 deficient mice developed an almost negligible response. Inhibition of the prostaglandin synthetizing enzyme COX by ibuprofen was effective in reducing the AITC-induced edema (Figure 6).

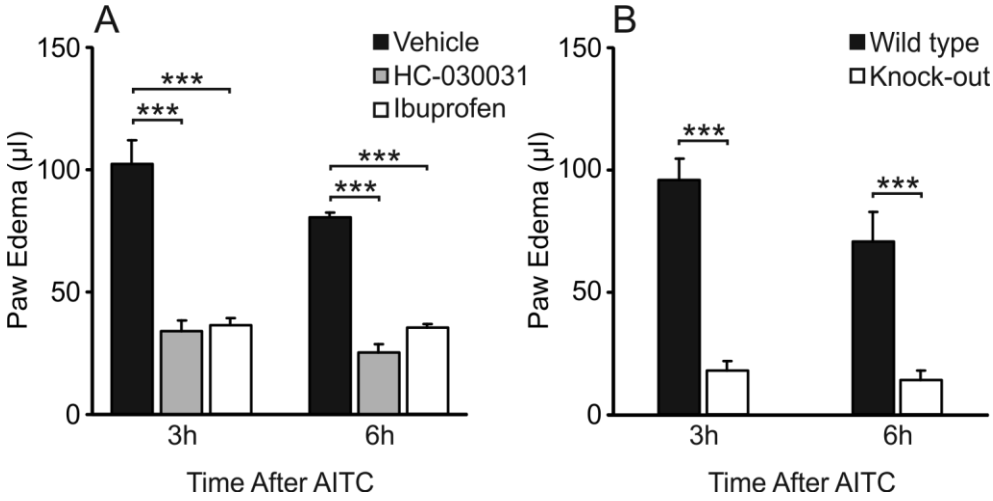


Figure 6. The effect of pharmacological inhibition and genetic depletion of TRPA1 on AITC-induced acute inflammatory paw edema. An intraplantar injection of the direct TRPA1 opener allyl isothiocyanate (AITC, 20 µg) induced an acute inflammatory paw edema inhibitable by treatment with the TRPA1 blocker HC-030031 (300 mg/kg) or the COX inhibitor ibuprofen (100 mg/kg) (A). Correspondingly, the AITC-induced edema was blunted in TRPA1 knock-out mice as compared to the corresponding wild type mice (B). Drugs were given intraperitoneally 2 h prior to the AITC injection and the edema was measured with a plethysmometer before and 3 h and 6 h after the AITC injection and compared to the basal level. The contralateral control paw injected with the vehicle developed no measurable edema. The results are expressed as mean + SEM, n=5-6, ***=p<0.001. Modified from Moilanen et al. Sci Rep, 2012; 2: 380.

4.1.3 AITC Increases COX-2 Expression in a TRPA1-Dependent Manner

As the ibuprofen treatment was effective in inhibiting the development of TRPA1-mediated edema, we were interested in studying the interaction of TRPA1 activation and expression of COX-2, which is the isoform of COX enzyme frequently induced in inflammation. Interestingly, the HEK 293 cells overexpressing TRPA1 showed an increase in COX-2 mRNA levels in the response to application of the TRPA1 opener AITC and this effect was inhibited by the TRPA1 blocker HC-030031. A similar response was absent in nontransfected HEK 293 cells (Figure 7).

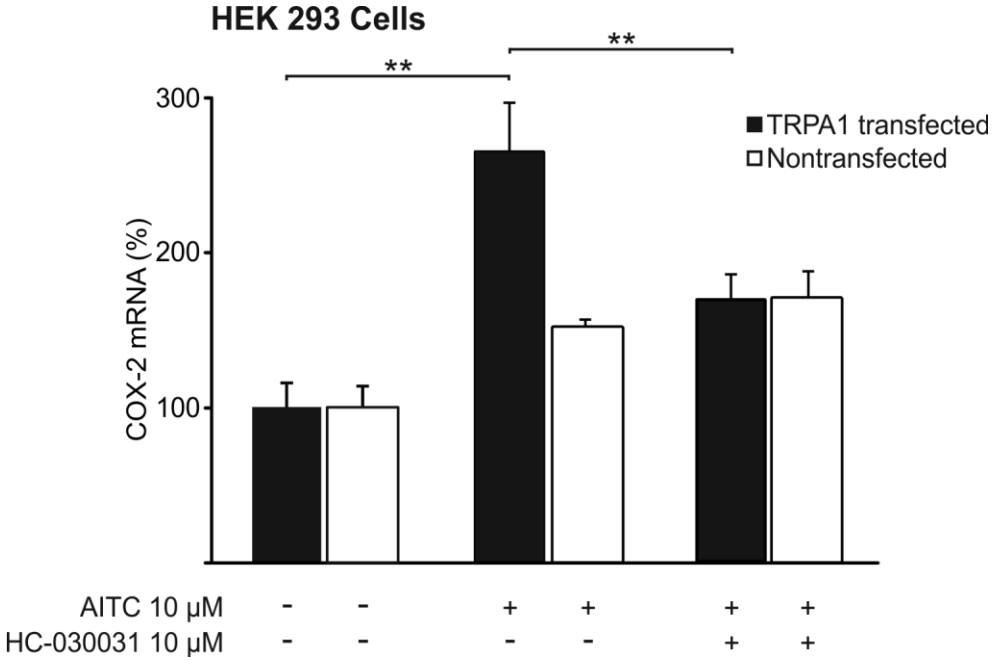


Figure 7. The effect of AITC on COX-2 expression in TRPA1 transfected and nontransfected HEK 293 cells. HEK 293 cells transfected with TRPA1 showed an upregulation of cyclooxygenase-2 (COX-2) mRNA in response to the TRPA1 opener allyl isothiocyanate (AITC). The expression was suppressed by application of the TRPA1 blocker HC-030031 given 30 min before AITC. The nontransfected cells did not exhibit TRPA1 upregulation in response to AITC stimulation. The cells were incubated with AITC for 6 h and then assayed for COX-2 mRNA by RT-PCR. COX-2 mRNA was normalized against GAPDH mRNA. The results are expressed as mean + SEM, n=4, **=p<0.01. Modified from Moilanen et al. Sci Rep, 2012; 2: 380.

4.1.4 Carrageenan is not a Direct TRPA1 Activator

As TRPA1 is highly susceptible to many external chemicals, an obvious explanation for the role of TRPA1 in the carrageenan-induced acute paw edema would be the direct activation of the ion channel by carrageenan. This was studied by Fura-2-AM assay by measuring Ca^{2+} influx in HEK 293 cells overexpressing TRPA1. Even a high concentration of carrageenan (250 μg/ml) failed to induce TRPA1 mediated Ca^{2+} influx whereas the positive control, AITC, evoked a robust response which was inhibited by treatment with HC-030031. This result indicates that carrageenan is not a direct TRPA1 activator and further places TRPA1 activation firmly in the cascade of inflammatory reaction.

4.2 TRPA1 in Experimental MSU Crystal-induced Gouty Inflammation (Study II)

4.2.1 TRPA1 Enhances MSU Crystal-Induced Gouty Inflammation

We combined the clear results attributing to TRPA1 a proinflammatory role in the carrageenan-induced paw edema and the previous knowledge of nociceptive and hyperalgesic properties of TRPA1 and based on this information, hypothesized that TRPA1 could well have a role in another experimental inflammatory disease model. We focused on an exceptionally painful and intense acute type of inflammation: gout flare. An experimental gouty inflammation was induced in the air-pouch test after the injection of MSU crystals. The analysis of subsequent production of proinflammatory cytokines revealed that treatment with the TRPA1 blocker HC-030031 was highly effective in reducing the total accumulation of inflammatory cells and cytokines MCP-1, MPO, MIP-1α and MIP-2 and a similar trend was seen in IL-6 and IL-1β (Figure 8A). Furthermore, a comparison of TRPA1 deficient and the corresponding wild type mice revealed attenuated amounts of inflammatory cells, IL-6, IL-1β, MPO, MIP-1α and MIP-2 as well as a trend towards a decrease in the accumulation of MCP-1 in TRPA1 deficient mice (Figure 8B).

A subcutaneous acute inflammation was triggered by injecting MSU crystals intraplantarly and the development of edema was measured. Interestingly, the edema evoked by MSU crystals was inhibited by the pre-treatment with the TRPA1 blocker HC-030031 and attenuated in the TRPA1 deficient mice when compared to their corresponding wild type counterparts (Figure 9A and 9B).

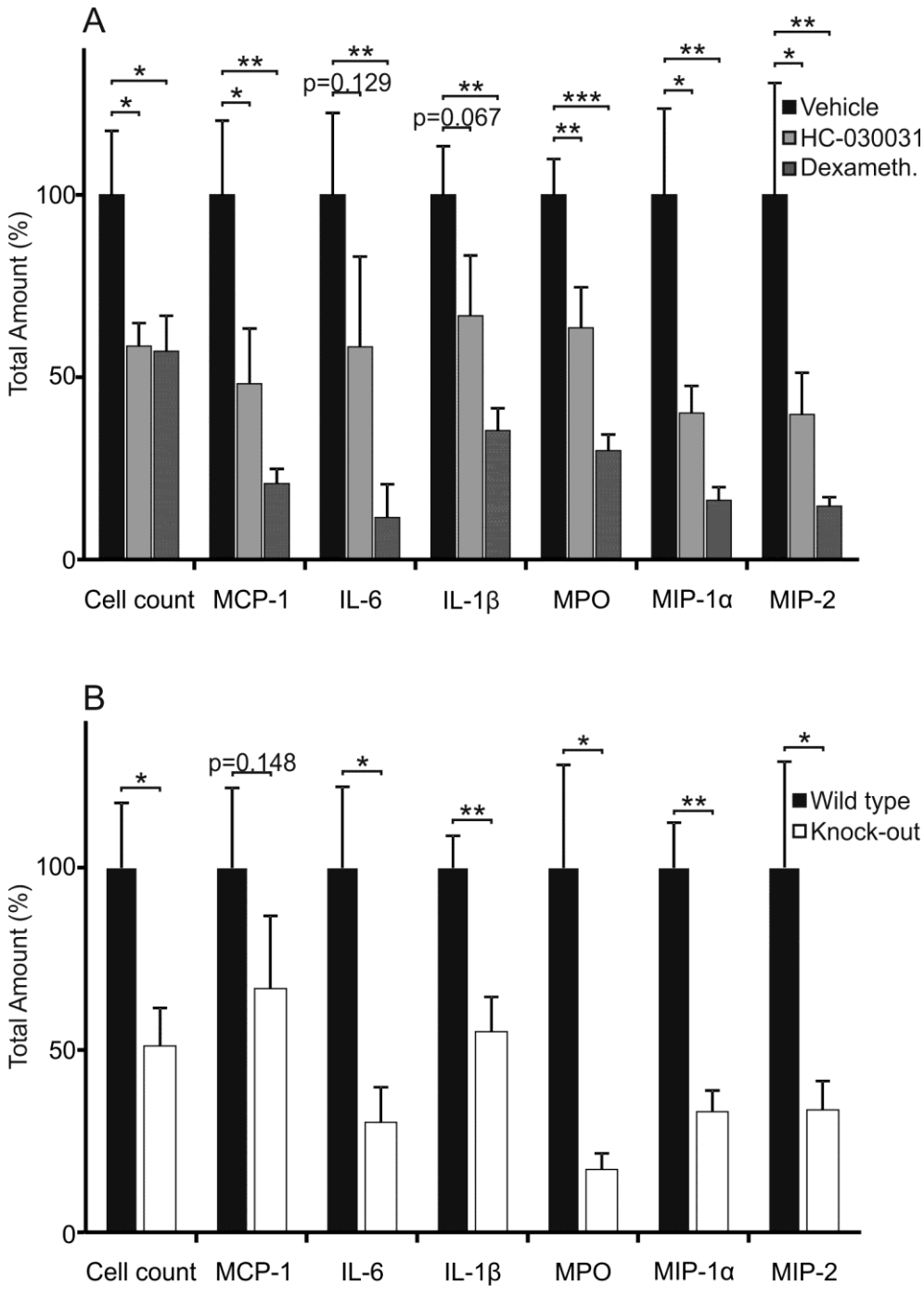

A
Total Amount (%)
100
50
0
p=0.129
p=0.067
Vehicle
HC-030031
Dexameth.
Cell count
MCP-1
IL-6
IL-1β
MPO
MIP-1α
MIP-2
B
Total Amount (%)
100
50
0
p=0.148
Wild type
Knock-out
Cell count
MCP-1
IL-6
IL-1β
MPO
MIP-1α
MIP-2

Figure 8. The effect of pharmacological inhibition and genetic depletion of TRPA1 on MSU crystal-induced inflammation in the air-pouch model. Treatment with the TRPA1 blocker HC-030031 (300 mg/kg) or dexamethasone (dexameth., 2 mg/kg) inhibited monosodium urate (MSU) crystal-induced production of proinflammatory cytokines MCP-1, IL-6, IL-1β, MPO, MIP-1α and MIP-2 and accumulation of inflammatory cells in the synovial joint resembling subcutaneous air-pouch model in the mouse (A). Accordingly, genetic depletion of TRPA1 led to an attenuated inflammatory response when compared to corresponding wild type mice (B). The studied drugs were given orally 2 h prior to 3 mg of MSU crystals suspended in 1 ml of endotoxin free sterile PBS were injected into the air-pouch. The exudate was harvested 6 h after the MSU crystal injection and cells were counted using hemocytometer and the cytokines were analysed using ELISA. Results are displayed as total amount of cells or cytokines per air-pouch. The mean amount of the vehicle treated mice was set as 100% to which the other values were related. The results are expressed as mean + SEM, n=7-8, *=p<0.05, **=p<0.01, ***=p<0.001. Modified from Moilanen et al. PLoS One, 2015; 10(2): e0117770.

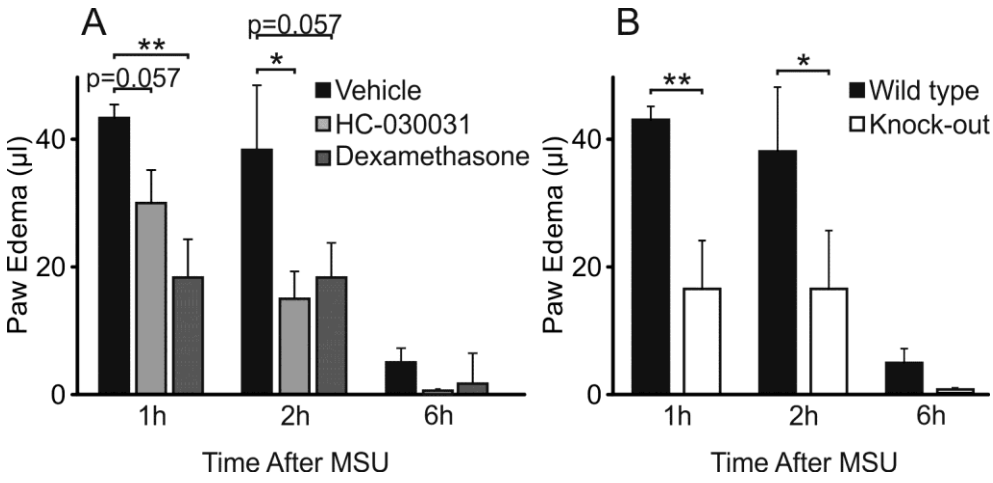


Figure 9. The effect of pharmacological inhibition and genetic depletion of TRPA1 on MSU crystal-induced inflammatory paw edema. Treatment with the TRPA1 blocker HC-030031 (300 mg/kg) or dexamethasone (2 mg/kg) inhibited mouse paw edema formation induced by an injection of monosodium urate (MSU) crystals (A). TRPA1 deficiency caused an alleviated edema formation compared to the corresponding wild type mice (B). The studied drugs were given orally 2 h prior to the initiation of the experiment by injecting 500 µg of MSU crystals in 40 µl of endotoxin free sterile PBS into the mouse hind paw. The paw volume was measured with a plethysmometer before and up to 6 h after MSU crystal injection. Paw edema is expressed as the volume change from the pre-treatment value and the results are displayed as mean + SEM, n=5-6, *=p<0.05, **=p<0.01. Modified from Moilanen et al. PLoS One, 2015; 10(2): e0117770.

4.2.2 TRPA1 Mediates Pain in Experimental Gouty Arthritis

As severe pain and hyperalgesia are very prominent in acute gout flare, we continued by attempting to assess whether TRPA1 would influence the gouty pain. In our experiments, the intra-articular injection of MSU crystals into the knee joint of the studied mice produced an acute painful arthritis as measured in the weight-bearing test, which is sensitive at assessing joint pain. The studied animals developed a clear reduction in spontaneous weight-bearing on the affected limb when measured 1, 2 and 3 days after the MSU crystal injection but the weight distribution had normalized on the 4th day. The TRPA1 deficient mice did not exhibit nociceptive behaviour and their weight distribution remained practically intact during the measurements (Figure 10).

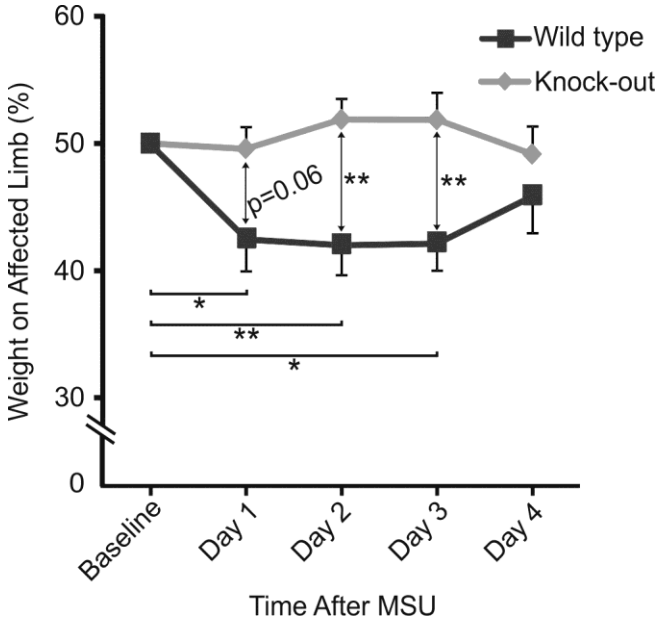


Figure 10. The effect of genetic depletion of TRPA1 on the response to MSU crystals in the spontaneous weight-bearing test. Spontaneous weight-bearing on knee joint injected with monosodium urate (MSU) crystals indicative of joint pain was altered in wild type but not in TRPA1 knock-out mice. The difference between the groups was also significant. An injection of MSU crystals (500 µg suspended in 40 µl of endotoxin-free sterile PBS) into the knee joint was performed and the mice were measured for the spontaneous distribution of weight between hind limbs with an incapacitance meter on four subsequent days and referred to the basal level. The contralateral knee was injected with the vehicle only and the investigator was blinded to the affected limb. Results are displayed as the percentage of weight borne by the affected limb and given as mean + SEM, n=7-10, *=p<0.05, **=p<0.01. Modified from Moilanen et al. PLoS One, 2015; 10(2): e0117770.

4.2.3 MSU Crystals do not Activate TRPA1 Directly

Direct activation of TRPA1 by MSU crystals was excluded by studying Ca^{2+} influx in TRPA1-transfected HEK 293 cells with the Fluo-3-AM assay. In the measurements, MSU crystals (1 mg/ml) did not evoke Ca^{2+} influx into the TRPA1-transfected cells, proposing that MSU crystals had not activated TRPA1 directly. In contrast, the TRPA1 channel opener AITC induced a robust increase in the intracellular Ca^{2+} concentration which could be inhibited by treatment with the TRPA1 blocker HC-030031.

4.3 Involvement of TRPA1 in MIA-Induced Experimental Osteoarthritis and Inflammation (Study III)

4.3.1 MIA-Induced Acute Inflammation is Partly Dependent on TRPA1 Activation

MIA-induced experimental osteoarthritis is a widely used *in vivo* model of osteoarthritis causing a similar joint degeneration as seen in human osteoarthritis. Based on the fact that MIA induces an acute inflammation including the production of ROS (Jiang et al., 2013), we hypothesized that TRPA1 activation would be involved in MIA-induced effects. Our initial experiments found evidence for TRPA1 activation in the acute inflammatory edema following an intraplantar injection of MIA. Treatment with the TRPA1 blocker, TCS 5861528, attenuated the development of acute inflammatory paw edema (Figure 11A). Similarly, the genetic depletion of TRPA1 resulted in a decreased development of edema (Figure 11B). Interestingly, the MIA-induced edema was blunted with inhibition of the putative endogenous TRPA1 openers by treatment with catalase which is a H_2O_2 degrading enzyme. The response was also attenuated by inhibiting TRPA1's secondary effector, substance P, by blocking its receptor with L703,606 (Figure 11C).

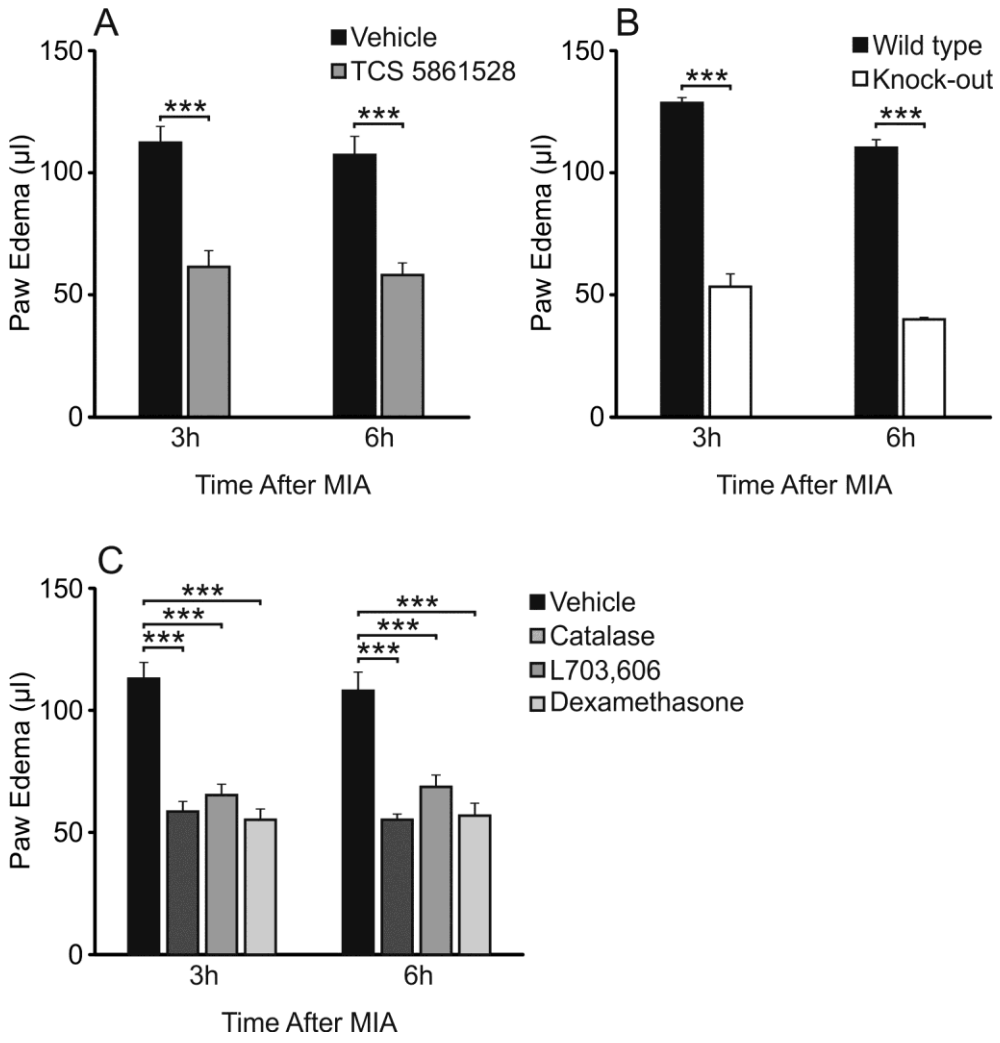


Figure 11. The effect of TRPA1 inhibition, catalase and substance P receptor antagonism on MIA-induced acute paw edema. Monosodium iodoacetate (MIA) induced an acute inflammatory edema when injected into the mouse hind paw. Treatment with the TRPA1 blocker TCS 5861528 (10 mg/kg) significantly inhibited the MIA-induced mouse paw edema (A). Accordingly, TRPA1 knock-out mice developed an alleviated edema in response to MIA as compared to the corresponding wild type mice (B). In addition, treatment with the H_2O_2 degrading enzyme, catalase (300 IU/paw) and neurokinin 1 receptor blocker L703,606 (10 mg/kg) inhibited the MIA-induced edema formation (C) similar to the TRPA1 blocker TCS 5861528. 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 up to 6 h after MIA injection (400 µg dissolved in 40 µl of endotoxin free sterile PBS). The results are expressed as mean + SEM, n=6-8, ***=p<0.001. Modified from Moilanen et al. Osteoarthritis Cartilage, 2015; 23(11): 2017-2026.

Further evidence for the role of TRPA1-induced release of substance P in the MIA-induced acute paw inflammation model was revealed in the analysis of the inflamed paw tissue. MIA injection increased the release of substance P into the inflamed paw tissue whereas negligible levels were measured from the contralateral tissue injected with the vehicle. Furthermore, TRPA1 deficient mice had an attenuated release of substance P as compared to the corresponding wild type mice in response to MIA injection (Figure 12A). Similarly, treatment with TCS 5861528 inhibited the release of substance P (Figure 12B).

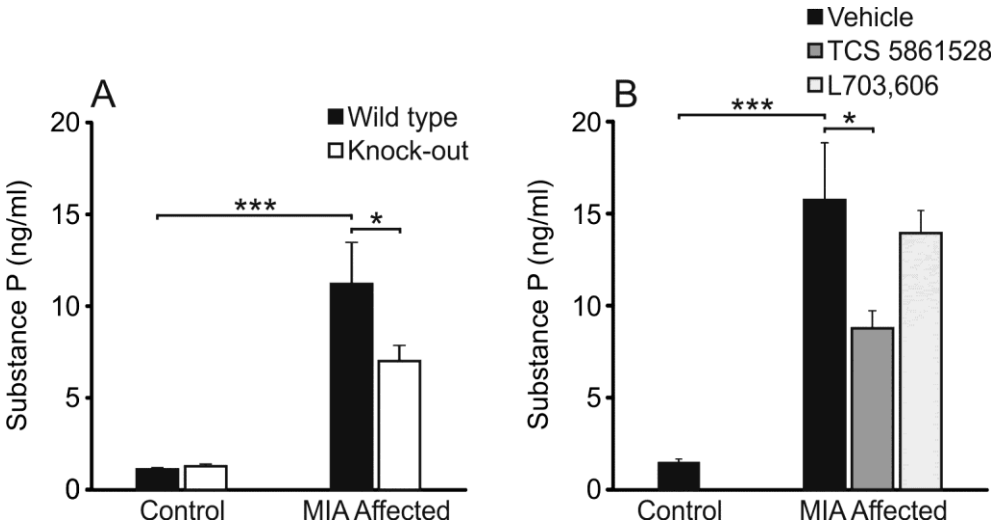
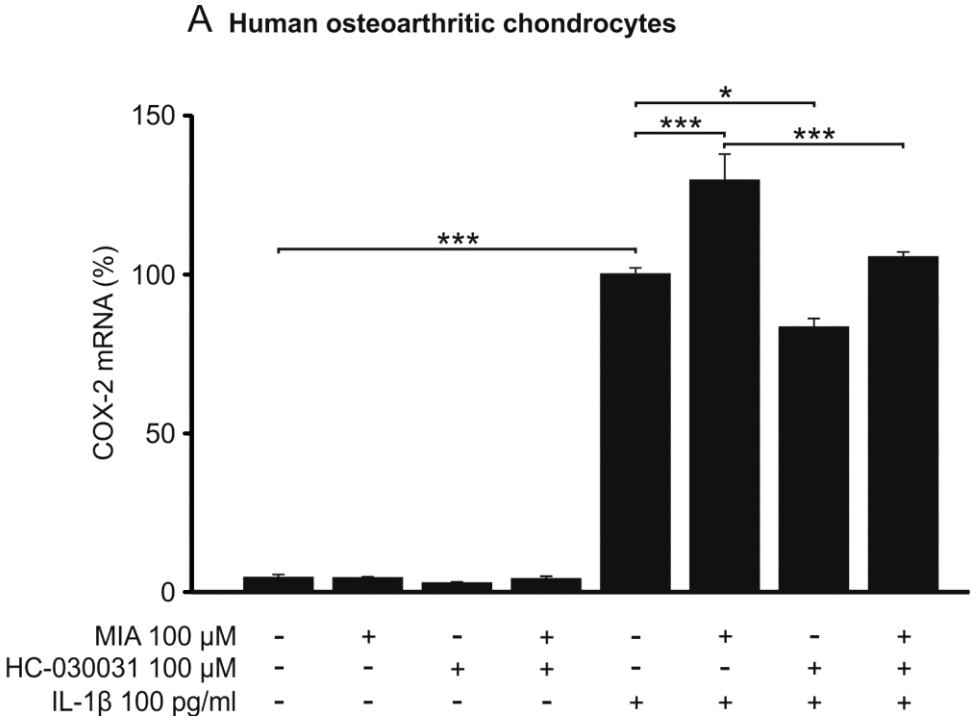


Figure 12. The effect of TRPA1 inhibition on MIA-induced release of neuropeptide substance P. Substance P concentrations were increased in the mouse paw following an injection of monosodium iodoacetate (MIA). Interestingly, substance P release was lower in TRPA1 knock-out mice as compared to the corresponding wild type mice (A). Accordingly, treatment with the TRPA1 blocker TCS 5861528 (10 mg/kg) inhibited the release of substance P whereas the neurokinin 1 receptor blocker 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 sterile PBS) whereas the contralateral control paw was injected with the vehicle. After 6 h, the mice were sacrificed and the collected paw tissue samples were analysed for substance P concentrations by ELISA. The results are expressed as mean + SEM, n=6-8, *=p<0.05, ***=p<0.001. Modified from Moilanen et al. Osteoarthritis Cartilage, 2015; 23(11): 2017-2026.

4.3.2 MIA Increases COX-2 Expression in Chondrocytes via TRPA1

Previously, increased COX-2 expression in the affected joints has been reported in the MIA-induced osteoarthritis (Dumond et al., 2004; J. Lee et al., 2013). Furthermore, the findings in the study I concerning the association of TRPA1 activation and COX-2 upregulation led us to hypothesize that this could also occur during the MIA-induced inflammatory reaction. Obviously, TRPA1 must be expressed in the studied cells before one can detect TRPA1-mediated effects. We confirmed by RT-PCR, that human chondrocytes indeed expressed TRPA1 and therefore proceeded with the *in vitro* experiments.

Next, we treated the cultured primary human osteoarthritic chondrocytes and naïve mouse cartilage tissue with IL-1β and MIA and found the COX-2 mRNA and protein levels to be highly increased. The effect of MIA was synergistic, meaning that MIA alone did not affect COX-2 expression, but when MIA was combined with IL-1β, the COX-2 expression increased as compared to the basal upregulation of COX-2 by IL-1β alone. Very interestingly, the effect of MIA on the COX-2 upregulation in IL-1β stimulated human chondrocytes was reversed with treatment with the TRPA1 blocker HC-030031 (Figure 13A). Furthermore, similar results were



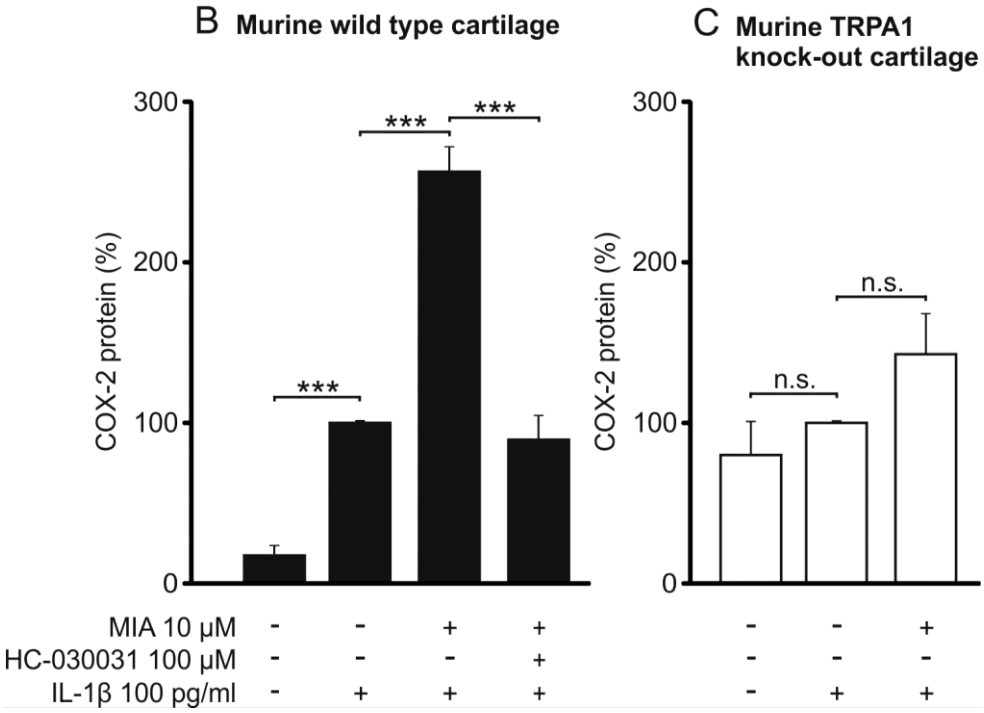
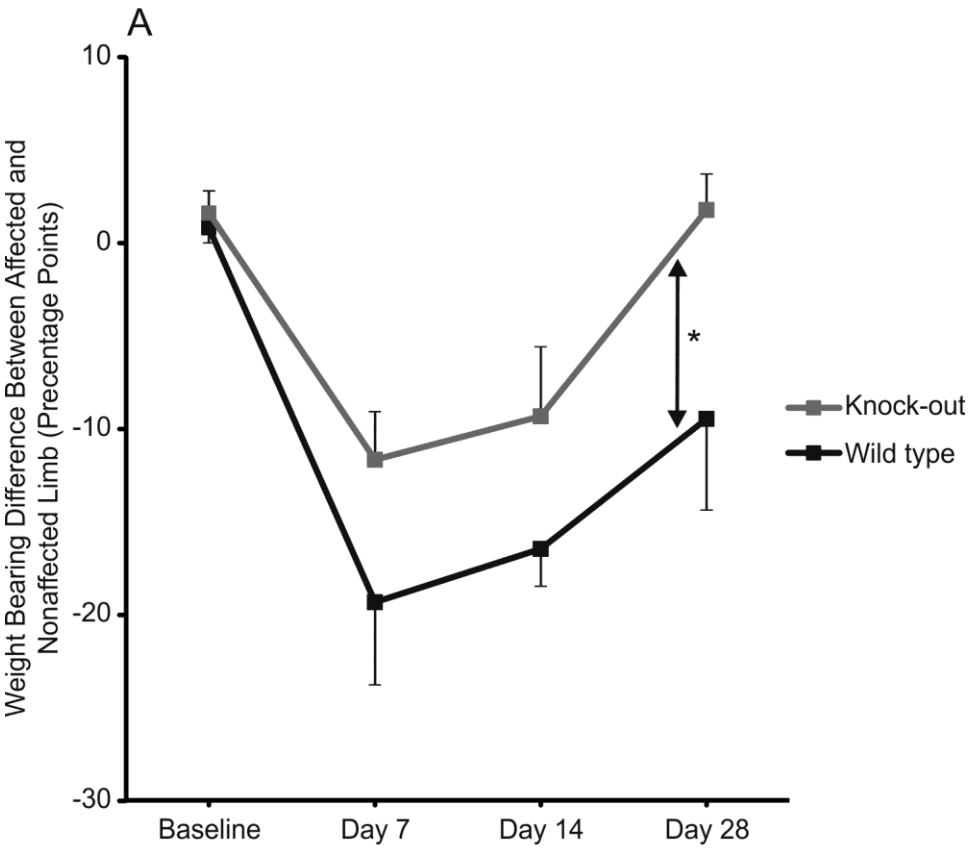


Figure 13. The effect of pharmacological inhibition and genetic depletion of TRPA1 on COX-2 expression in cultured human osteoarthritic chondrocytes and murine cartilage. In primary cultures of human osteoarthritic chondrocytes, treatment with the TRPA1 blocker HC-030031 reduced the expression of proinflammatory gene cyclooxygenase-2 (COX-2) induced by co-stimulation with monosodium iodoacetate (MIA) and interleukin-1β (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, C). The samples were cultured together with the studied compounds for 6 h and analysed for COX-2 mRNA expression by RT-PCR (A) or for 24 h and analysed for COX-2 protein expression by Western Blotting (B, C). The mRNA expression was normalized against the housekeeping gene, GAPDH (A) and the protein expression against the loading control, actin (B, C). The response in IL-1β treated chondrocytes / cartilage was set at 100% and the other results are expressed in relation to that value. The human samples were obtained from 6 different donors and the experiments were performed in triplicate (A). The cartilage samples were from five mouse (n=5) in each treatment (B, C). The results are expressed as mean + SEM, n=6-8, *=p<0.05, ***=p<0.001, n.s.=not significant. Modified from Moilanen et al. Osteoarthritis Cartilage, 2015; 23(11): 2017-2026.


A
10
0
-10
-20
-30
Weight Bearing Difference Between Affected and Nonaffected Limb (Precentage Points)
Baseline
Day 7
Day 14
Day 28
*
Knock-out
Wild type

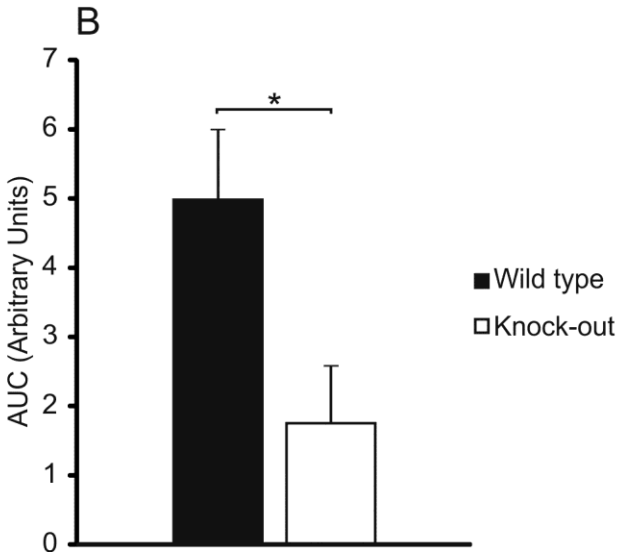
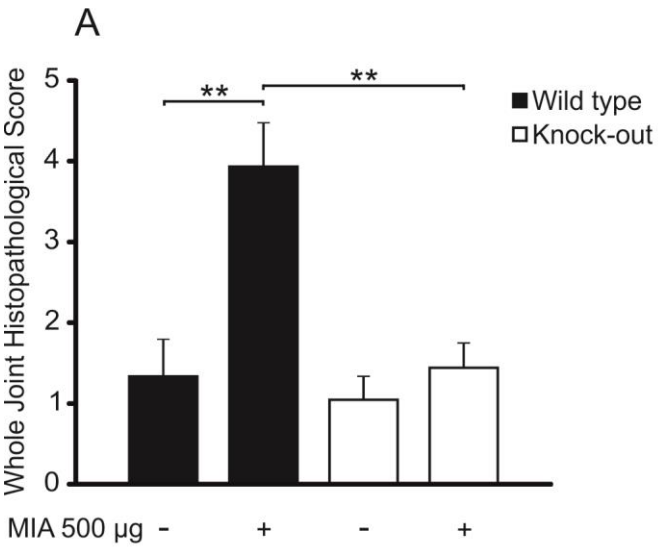

B
7
6
5
4
3
2
1
0
AUC (Arbitrary Units)
*
Wild type
Knock-out

Figure 14. The response of TRPA1 deficient mice to MIA in the weight-bearing test. An injection of monosodium iodoacetate into the knee joint developed a reduction in spontaneous weight-bearing on the affected limb indicative of joint pain. Interestingly, an attenuated weight distribution change was seen in TRPA1 knock-out mice. Spontaneous distribution of weight between the hind limbs was measured with an incapacitance meter before and 7, 14 and 28 days after intra-articular injection of MIA (500 µg dissolved in 40 µl of endotoxin-free sterile PBS). The contralateral knee joint was injected with the vehicle and the investigator was blinded to the affected limb. In the upper picture (A), the results are displayed as the difference of percentage points between the weight borne by the affected and non-affected limbs and the statistical analysis was carried out between the groups on the whole curve. In the second picture (B), area-under-the curve (AUC) for the shift from the equal balance between the hind limbs was calculated. The results are expressed as mean + SEM, n=7, *=$p<0.05$. Modified from Moilanen et al. Osteoarthritis Cartilage, 2015; 23(11): 2017-2026.

observed in the experiments conducted in cultured murine cartilage. In wild type tissue, the increase in COX-2 protein production evoked by addition of MIA to IL-1β stimulation was reversed by HC-030031 (Figure 13B). Correspondingly, in TRPA1 deficient tissue, the combination of MIA to IL-1β did not induce an increase in the COX-2 protein expression (Figure 13C). Collectively, the experiments in cultured human osteoarthritic chondrocytes and murine cartilage demonstrated that MIA increased the COX-2 expression when it was added together with IL-1β and this effect was dependent on TRPA1 activation.

4.3.3 TRPA1 Mediates Cartilage Degeneration and Joint Pain in MIA-Induced Osteoarthritis

As it seemed highly likely that TRPA1 activation is crucial for the initial inflammation induced by MIA, we were interested to know if TRPA1 is also associated with the development of joint pain and histopathological changes during the course of MIA-induced experimental osteoarthritis. In the weight bearing test, an intra-articular injection of MIA induced a weight distribution away from the affected limb, indicative of joint pain during the 28-day follow-up. Interestingly, the TRPA1 deficient mice had a reduced response to MIA-injection as their weight shift was smaller than in the corresponding wild type mice (Figure 14A and 14B). Furthermore, after the weight-bearing test, the joints were dissected and analysed histologically for cartilage degeneration. The results showed that the changes in wild type mice cartilage exceeded those in TRPA1 deficient mice proposing a role for TRPA1 in the development of MIA-induced osteoarthritis (Figure 15A and 15B).


A
Whole Joint Histopathological Score
5
4
3
2
1
0
**
**
Wild type
Knock-out
MIA 500 µg -
+
-
+

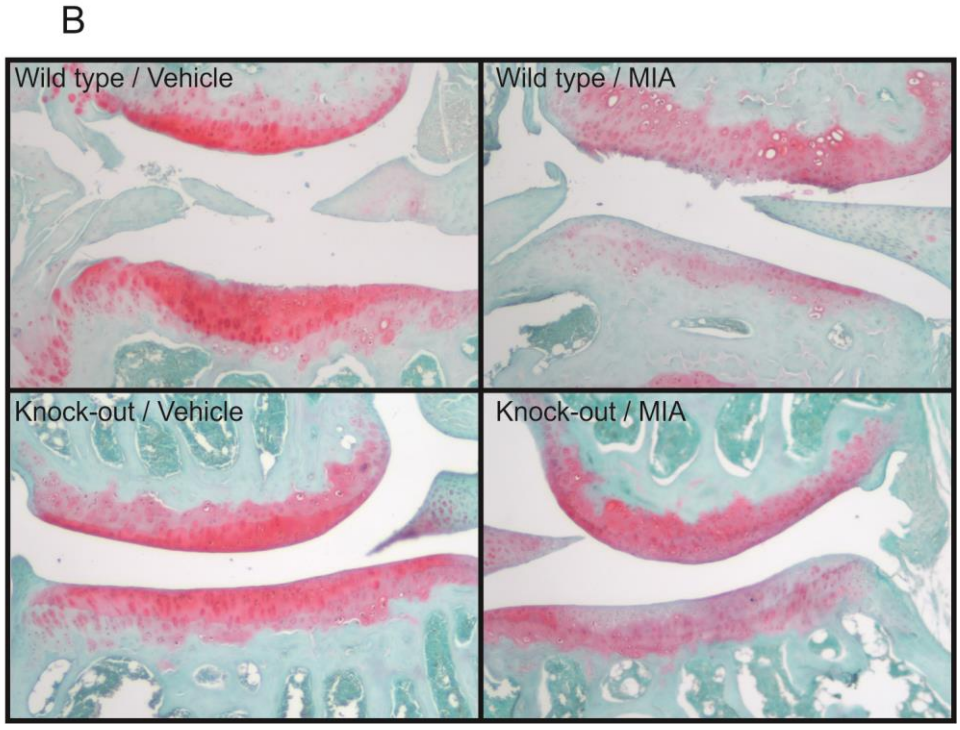

B
Wild type / Vehicle
Wild type / MIA
Knock-out / Vehicle
Knock-out / MIA

Figure 15. The development of histopathological changes in TRPA1 deficient mice in MIA-induced experimental osteoarthritis. When monosodium iodoacetate (MIA) was injected into the knee joint, wild type mice developed more severe cartilage changes than TRPA1 knock-out mice. Twenty-eight days after intra-articular injection of MIA (500 µg dissolved in 40 µl of endotoxin-free sterile PBS) into one knee joint and the vehicle into the contralateral joint, mice were sacrificed and the knee joints were dissected for histology. The cartilage changes were scored according to the OARSI guidelines (Glasson et al., 2010). In the upper figure (A), the whole joint histopathological score is presented. In the lower image (B), representative figures of MIA-injected and vehicle-injected (control) contralateral knee joints from wild type and TRPA1 knock-out mice are shown (magnification x10). The results are expressed as mean + SEM, n=7, **=p<0.01. Modified from Moilanen et al. Osteoarthritis Cartilage, 2015; 23(11): 2017-2026.

4.3.4 MIA is not a Direct TRPA1 Activator

As in the earlier studies, we were interested if the stimulant, i.e. MIA, acted through direct TRPA1 activation. The Fluo-3-AM measurements found no evidence of MIA induced Ca^{2+} influx in HEK 293 cells expressing TRPA1 while the positive control compound, AITC, induced a rapid Ca^{2+} influx which could be antagonized by the TRPA1 blocker HC-030031.

4.4 TRPA1 in Ovalbumin-Induced Acute Allergic Inflammation (Study IV)

4.4.1 Activation of TRPA1 is Crucial in the Development of Ovalbumin-Induced Allergic Conjunctivitis

Inspired by previous findings on the ovalbumin-induced airway hyperreactivity (Caceres et al., 2009), we investigated the relationship between TRPA1 and allergen-induced acute conjunctivitis. In our experiments, the studied mice were sensitized to ovalbumin and then challenged topically via the eye. The developed conjunctivitis was analysed after 24 h by measuring the infiltration of eosinophils. The results showed that the local treatment with the TRPA1 blocker, TCS 5861528, clearly inhibited the accumulation of eosinophils into the conjunctiva similar to the situation in mice with genetic depletion of TRPA1 (Figure 16A and 16B).

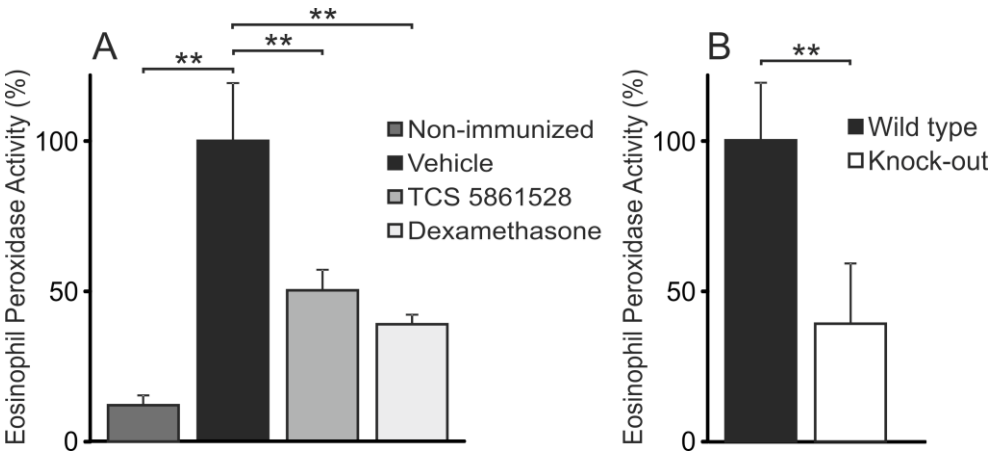


Figure 16. The effect of TRPA1 inhibition on ovalbumin-induced allergic conjunctivitis. The conjunctivitis induced by topical application of ovalbumin was inhibited by treatment with the TRPA1 blocker TCS 5861528 (25 µg suspended in 5 µl of sterile PBS) or the anti-inflammatory control compound dexamethasone (5 µg in 5 µl, commercial product) (A) when measured by the eosinophil peroxidase activity in the tissue reflecting the amount of infiltrated eosinophils. Correspondingly, the TRPA1 knock-out mice exhibited an alleviated conjunctivitis as compared to the corresponding wild type mice (B). The conjunctivitis was induced in mice sensitized towards ovalbumin by topical application of ovalbumin (500 µg suspended in 5 µl of sterile PBS) into both eyes. The studied drugs were given topically at 1 h before and 2 h, 4 h and 6 h after the application of ovalbumin. At 24 h after the application of ovalbumin, the mice were sacrificed and conjunctiva from both eyes were surgically removed and analysed for eosinophil infiltration by measuring eosinophil peroxidase activity. The results are expressed as mean + SEM, n=5-6, **=p<0.01.

4.4.2 TRPA1 does not Affect Eosinophil Apoptosis

Apoptosis of tissue migrated eosinophils, for example in allergic conjunctivitis, is a physiological anti-inflammatory regulatory mechanism and dysfunction of eosinophil apoptosis is regarded as proinflammatory. Accordingly, eosinophil apoptosis is delayed in allergic inflammation through the effect of IL-5 and other allergy-associated cytokines (Ilmarinen et al., 2014). Furthermore, one of the most potent anti-inflammatory effects of glucocorticoids in allergic inflammation is the

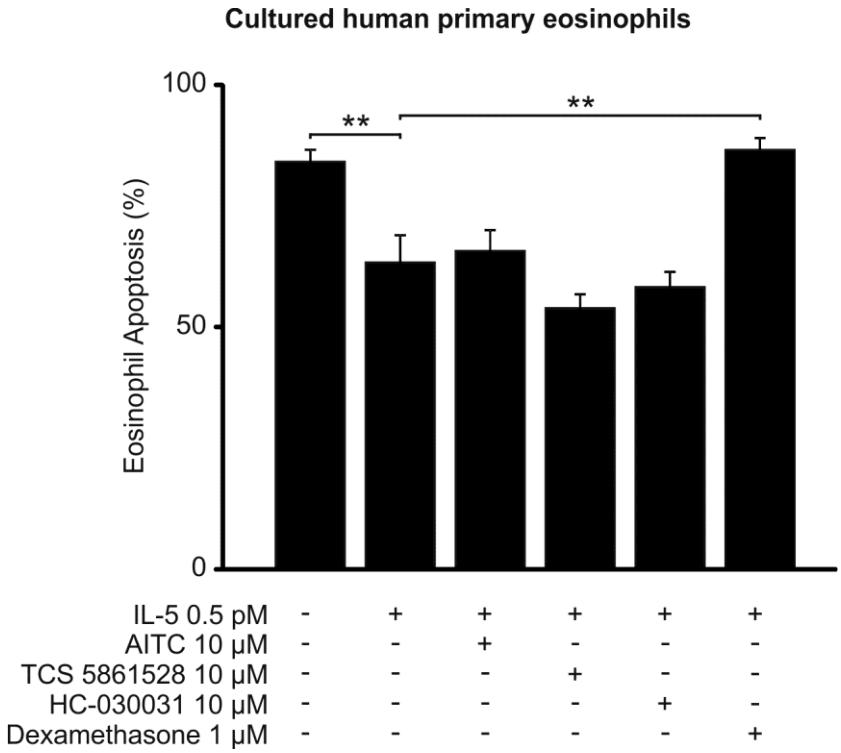


Figure 17. The effects of TRPA1 opener and blockers on spontaneous eosinophil apoptosis. The TRPA1 opener allyl isothiocyanate (AITC), or the TRPA1 blockers TCS 5861528 or HC-030031 did not have an impact on the IL-5 promoted eosinophil survival, whereas the anti-inflammatory glucocorticoid dexamethasone reversed the effect of IL-5 on the eosinophil apoptosis. The blood derived human primary eosinophils where cultured with the studied compounds for 40 h and eosinophil apoptosis was determined by using the relative DNA fragmentation assay and expressed as percentage of apoptotic cells. The blood was derived from 6 different donors and the experiments were performed with n=4.The results are expressed as mean + SEM, **=p<0.01.

driving of eosinophils towards apoptosis (Ilmarinen et al., 2014). Therefore we decided to explore the effect of TRPA1 openers and blockers on this mechanism. However, addition of either the TRPA1 opener AITC or the TRPA1 blocker HC-030031 or TCS 5861528 did not have an effect on this process (Figure 17). These findings suggest that the proinflammatory properties of TRPA1 in acute allergic inflammation are mediated by a distinct mechanism.

4.4.3 Ovalbumin-Induced Acute Paw Inflammation Involves TRPA1 Activation

In addition to the late-phase of the acute allergic inflammation measured by infiltration of eosinophils in the allergic conjunctivitis, we were interested in the early events of the reaction triggered by the allergen challenge. Hence, we applied ovalbumin subcutaneously into the paw of sensitized animals and followed the edema development for up to 6 h. The treatment with the TRPA1 blocker TCS 5861528 reduced the development of acute inflammatory paw edema and similar results were detected in mice treated with L703,606, an inhibitor of the substance P target receptor (Table 7A). Furthermore, the involvement of TRPA1 was supported by the findings showing that TRPA1 deficient mice had an impaired edema development in response to ovalbumin challenge (Table 7B).

After the paw edema test, the inflamed tissue was analysed for IL-4, a cytokine closely associated with acute allergic inflammation. The paw tissue from mice treated with the vehicle showed clearly increased production of IL-4 at the site of allergen challenge, but this effect was significantly reduced in mice treated with TCS 5861528 or L703,606 (Figure 18A). A similar outcome was seen in the comparison between TRPA1 deficient and wild type mice in which the levels measured in TRPA1 depleted animals were significantly reduced (Figure 18B). Together with the findings in allergic conjunctivitis, the results from the acute ovalbumin-induced paw inflammation indicate that TRPA1 activation is indeed important for the development of the allergen-induced acute inflammation. Furthermore, the effectiveness of the treatment with L703,606 indicates that the neuropeptide substance P is most likely a putative secondary effector of TRPA1.

Table 7. The effect of TRPA1 inhibition and substance P receptor antagonism on ovalbumin-induced acute paw edema. An intraplantar injection of ovalbumin (45 µg diluted in 45 µl of endotoxin free sterile PBS) induced an acute inflammatory paw edema that was inhibitable by treatment with the TRPA1 blocker TCS 5861528 (10 mg/kg), the blocker of the NK1 receptor (the receptor of substance P) L703,606 (10 mg/kg) or the anti-inflammatory control compound dexamethasone (2 mg/kg) (A). Similarly, the ovalbumin-induced edema was blunted in TRPA1 knock-out mice as compared to the corresponding wild type mice (B). The mice were sensitized towards ovalbumin and the drugs were given intraperitoneally 1 h prior to the ovalbumin injection and the edema was measured with a plethysmometer before and up to 6 h after treatment and compared to the basal level. The contralateral control paw injected with vehicle developed no measurable edema. The area under curve (AUC) was calculated for the entire experiment. The results are expressed as mean ± SEM, n=6-8, *=p<0.05, **=p<0.01, ***=p<0.001.

A

Treatment	Paw edema increase from baseline (µl)						AUC
	1 h	2 h	3 h	4 h	5 h	6 h	
Vehicle	57.5±4.9	48.8±3.7	36.3±1.8	36.3±4.2	27.5±1.6	17.5±2.6	215.0±12.9
TCS 5861528	40.0±5.8	33.3±3.0	28.3±2.8	18.3±3.7	15.0±3.1	8.3±4.4	140.8±14.6 *
L703,606	35.0±3.9	33.3±3.0	16.7±5.6	10.0±2.4	5.0±3.1	6.7±3.0	103.3±11.2 **
Dexamethasone	38.8±3.4	25.0±3.4	16.7±3.5	18.3±6.1	11.7±6.7	6.7±1.8	113.3±23.7 **

B

Genotype	Paw edema increase from baseline (µl)						AUC
	1 h	2 h	3 h	4 h	5 h	6 h	
Wild type	66.7±5.1	90.0±6.3	38.3±6.4	41.7±6.4	30.0±5.2	15.0±2.0	274.2±13.6
TRPA1 knock-out	37.5±3.4	31.3±4.8	30.0±5.8	18.8±2.1	10.0±4.0	8.8±3.3	131.9±13.3 ***

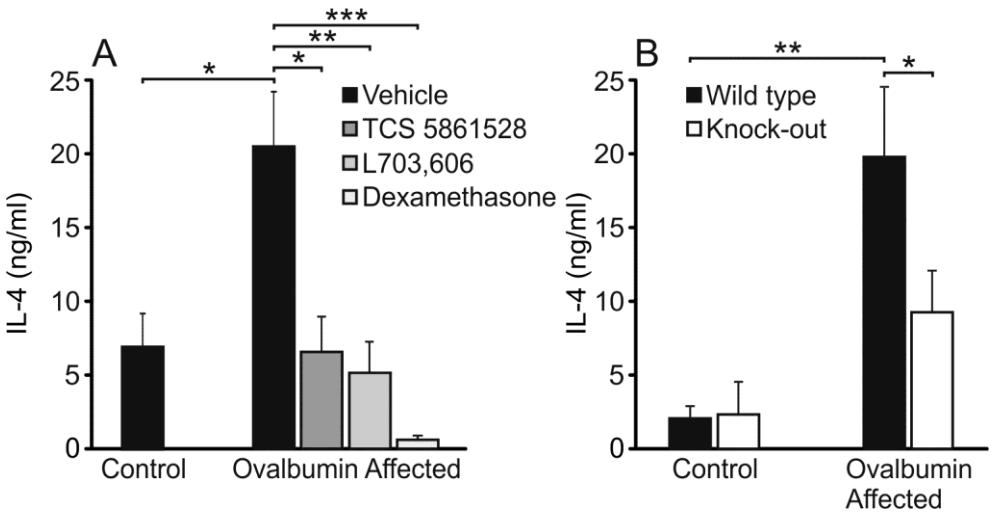


Figure 18. The effect of TRPA1 inhibition and substance P receptor antagonism on ovalbumin-induced IL-4 production. An intraplantar injection of ovalbumin (45 µg diluted in 45 µl of endotoxin free sterile PBS) induced an increase in the production of allergy-associated cytokine IL-4 inhibitable by treatment with the TRPA1 blocker TCS 5861528 (10 mg/kg), a blocker of NK1 receptor (the receptor of substance P) L703,606 (10 mg/kg) or the anti-inflammatory control compound dexamethasone (2 mg/kg) (A). Correspondingly, the increase of IL-4 production was blunted in TRPA1 knock-out mice as compared to the corresponding wild type mice (B). The mice were sensitized towards ovalbumin and the drugs were given intraperitoneally 1 h prior to the ovalbumin injection. After 6 h, the inflamed subcutaneous paw tissue and the paw injected with the solvent from the vehicle treated mice (control) were dissected and the concentrations of IL-4 were analysed by ELISA. The results are expressed as mean + SEM, n=6-8, *=p<0.05, **=p<0.01, ***=p<0.001.

4.4.4 Splenocytes in TRPA1 Deficient Mice Respond Normally to Ovalbumin

Sensitized TRPA1 deficient mice have normal levels of ovalbumin specific IgE antibodies, which is interpreted to mean that there was normal sensitization towards ovalbumin (Caceres et al., 2009). This is an interesting aspect with respect to the acute allergic inflammation as an impaired sensitization towards the allergen would obviously result in a milder reaction. We aimed at studying the sensitization from another angle and examined the splenocytes of the mice sensitized to ovalbumin by stimulating the isolated splenocytes with ovalbumin *in vitro* and by measuring the production of cytokine IL-13. First, the splenocytes isolated from mice which had

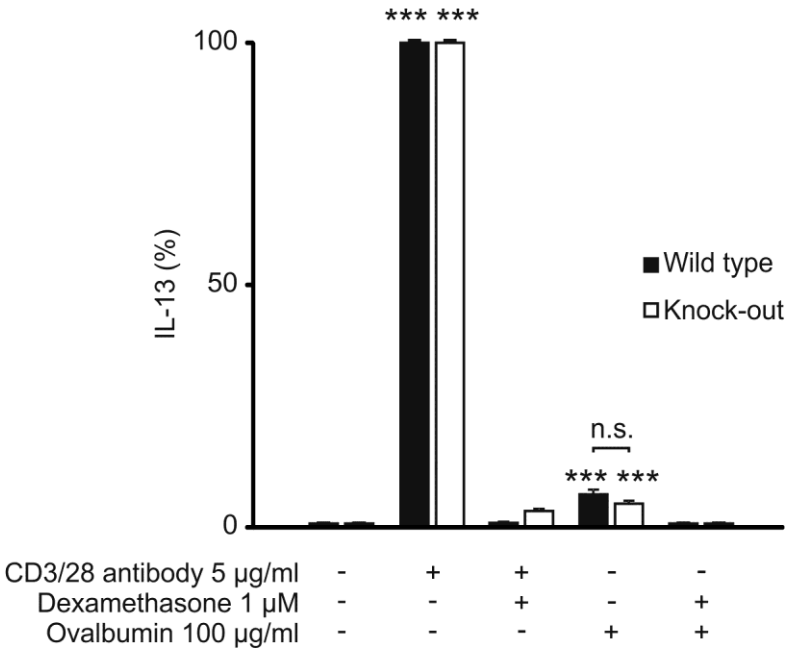


Figure 19. The splenocytes' response to ovalbumin in TRPA1 deficient and wild type mice. Isolated splenocytes derived from TRPA1 knock-out and wild type mice responded similarly to ovalbumin stimulation. The mice were sensitized to ovalbumin, euthanized and the spleens were surgically removed. The isolated splenocytes were cultured with the studied compounds for 42 h. Combibation of CD3 and CD28 antibodies (CD3 coated on the culture plate, CD28 diluted in culture medium) was used as a common T-cell activator to induce a robust IL-13 production and the results are proportional to that value. The results are expressed as mean + SEM, n=6, ***=p<0.001 as compared to the untreated group.

not been sensitized were totally inert to the stimulation with ovalbumin. However, the splenocytes from sensitized mice produced IL-13 in response to ovalbumin stimulation, indicative of successful sensitization. However, the TRPA1 deficiency did not affect the amount of IL-13 produced when the IL-13 levels were compared to the stimulation with T-cell activating CD3 and CD28 antibodies which induced a robust production of IL-13 (Figure 19).

4.4.5 Ovalbumin does not Activate TRPA1 Directly

As TRPA1 activation is clearly involved in the development of ovalbumin-induced acute allergic inflammation, we posed the question whether this would be mediated by direct activation of TRPA1 by ovalbumin. In agreement with our working

hypothesis, stimulation of TRPA1 transfected HEK 293 cells with ovalbumin did not evoke TRPA1 mediated Ca^{2+} influx. The positive control AITC induced a robust increase in intracellular Ca^{2+} concentration that was inhibited by the TRPA1 blocker HC-030031. These data indicate that ovalbumin is not a direct TRPA1 channel opener but most likely induces a release of endogenous TRPA1 activators which are responsible for the TRPA1 mediated effects of ovalbumin.

4.5 Pinosylvin as a Novel TRPA1 Blocker (Study V)

4.5.1 Pinosylvin Inhibits TRPA1 Activation *in Vitro*

The positive effects of inhibition of TRPA1 in many inflammatory models in the first four studies gave rise to the decision to search for TRPA1 inhibiting compounds. We decided to determine whether members of the stilbenoid group would be able to inhibit TRPA1 based on our preliminary screening experiments and on earlier findings showing that resveratrol inhibited TRPA1 (Yu et al., 2013). Pinosylvin, a naturally occurring stilbenoid, was tested in the Fluo-3-AM assay and it was found to inhibit TRPA1 activation induced by the TRPA1 channel opener, AITC, in a dose-dependent manner (IC_{50} 26.5 μM and Hill Slope -0.77) (Figure 20A and 20B). The results were uniform with the findings from a second membrane current assay, i.e. patch clamp. The results in patch clamp recordings showed that pinosylvin reversed the effect of AITC on membrane currents (IC_{50} 16.7 μM and Hill Slope -1.17) (Figure 20D and 20G). The effect of pinosylvin was very close to that obtained with resveratrol (IC_{50} 12.9 μM and Hill Slope -0.99) (Figure 20C and 20G). High concentrations of pinosylvin or resveratrol were capable of inducing a minor TRPA1-dependent ion influx, indicating that at high concentrations, these compounds could be TRPA1 activators (Figure 20E, 20F and 20H). Indeed, as described in the Review of the Literature section, some compounds exert bimodal effects on TRPA1, usually the effect has been described as being activation at low concentrations and inhibitory at higher concentrations.

4.5.2 Pinosylvin Inhibits TRPA1-Induced Inflammation *in Vivo*

Paw edema inflicted by an injection of the TRPA1 opener AITC into the paw of an experimental animal is regarded as a TRPA1-specific effect. Therefore we tested the

effect of pinosylvin *in vivo* in the AITC-induced paw edema model. The results showed that systemic treatment with pinosylvin was highly effective in decreasing the formation of paw edema (Figure 21A). Furthermore, the analysis of the inflamed paw tissue revealed that pinosylvin also inhibited the production of the powerful proinflammatory cytokine, IL-6, at the site of the inflammation (Figure 21B). Hence, pinosylvin is a promising candidate for drug development in the search for TRPA1 blockers.

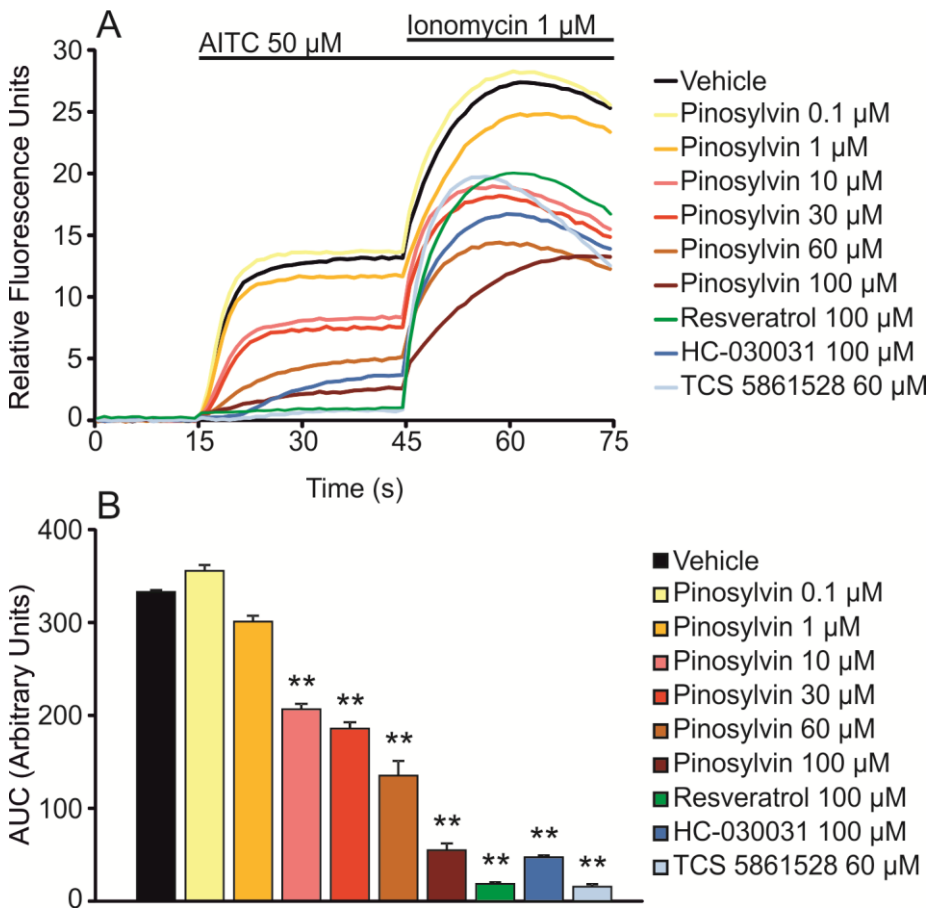
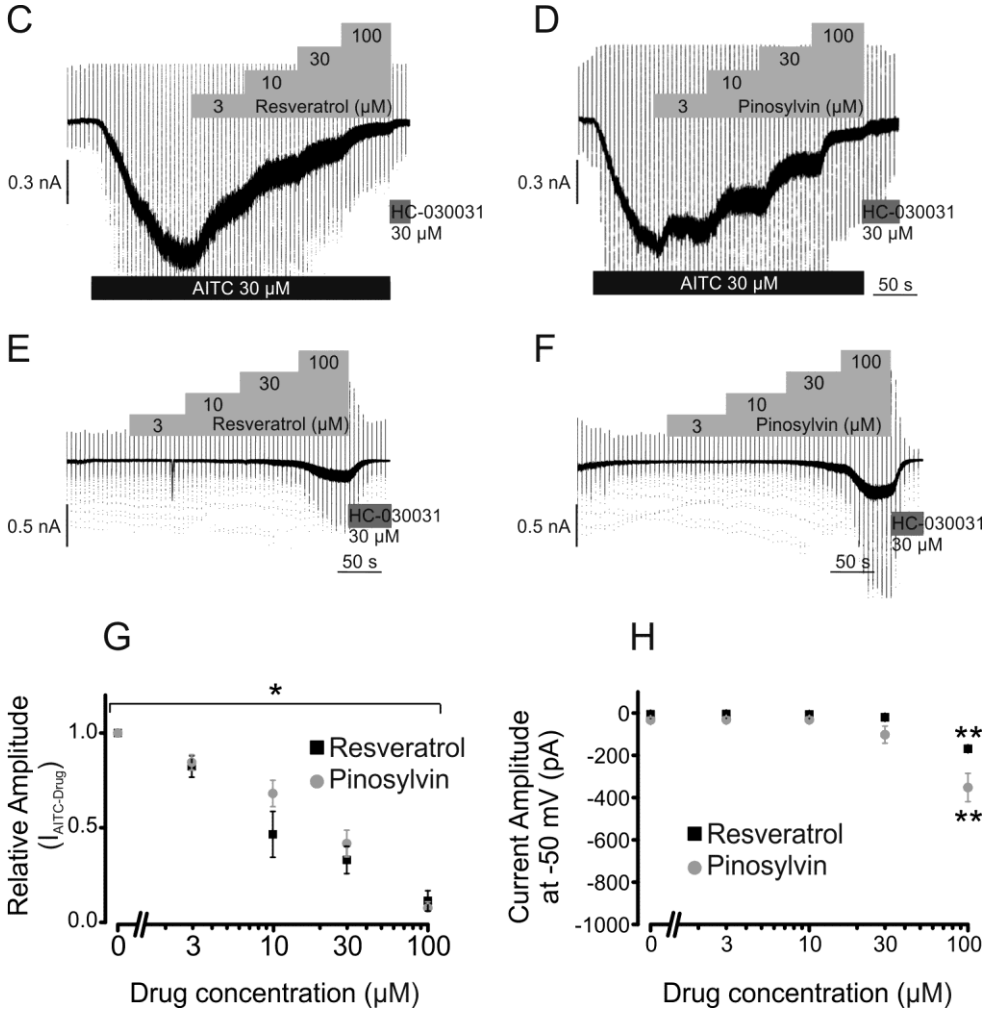


Figure 20. The effect of pinosylvin on TRPA1-mediated Ca^{2+} currents. In HEK 293 cells transfected with TRPA1, the treatment with pinosylvin dose-dependently abolished the Ca^{2+} influx current induced by the TRPA1 opener allyl isothiocyanate (AITC) and measured by the Fluo-3-AM assay (A and B). Similar inhibition was achieved by treatment with the known TRPA1 blockers TCS 5861528 and HC-030031 and also by treatment with resveratrol (A and B). In the first two figures, the cells were loaded with Fluo-3 AM and the intracellular Ca^{2+} concentration was measured at excitation/emission wavelengths of 485/535 nm at a 1/s frequency (A and B). The cells were first pre-incubated with the studied compounds or the vehicle for 30 min at 37 °C. In these experiments, the basal fluorescence was first measured for 15 s and thereafter AITC (50 µM) was added and the measurement was continued for 30 s after which the control ionophore compound ionomycin (1 µM) was applied to the cells. In the second figure, the area under curve (AUC) for the duration of AITC stimulation, i.e. the AUC value between time points 15 s and 45 s, was calculated and expressed as mean + SEM (B).



In whole cell patch clamp measurements, resveratrol and pinosylvin reversed dose-dependently the AITC-induced peak membrane currents when measured in HEK 293 cells transfected with TRPA1 (C, D). The results ($I_{AITC\text{-}Drug}$) are normalized against AITC-induced peak currents without resveratrol and pinosylvin (I_{AITC}), and expressed as mean± SEM (G). Finally, a high concentration of resveratrol and pinosylvin also induced minor TRPA1-mediated membrane currents measured correspondingly by patch clamp in similarly transfected cells (E, F). AITC-induced peak currents with or without resveratrol and pinosylvin are expressed as mean± SEM (H). n=4-6, **=p<0.01. Modified from Moilanen et al. Basic Clin Pharmacol Toxicol, 2016; 118(3): 238-242.

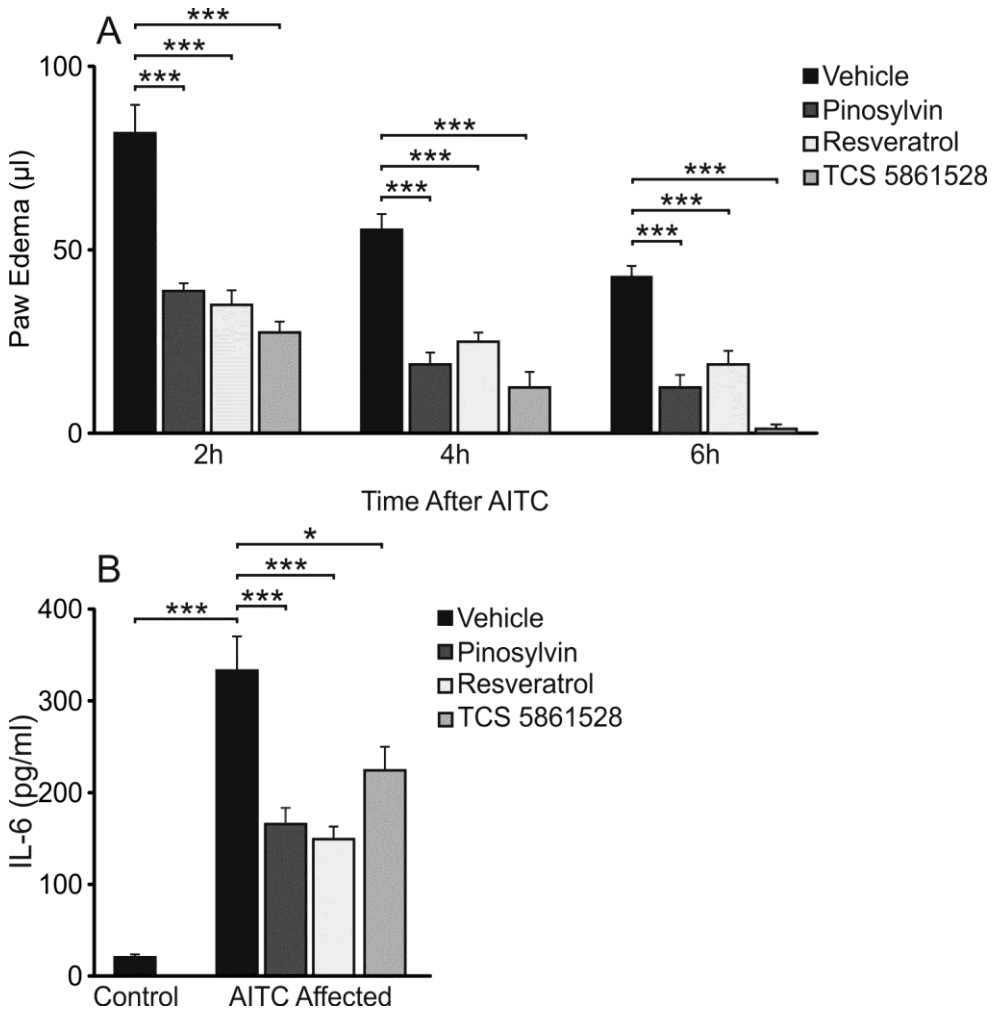


Figure 21. The effect of pinosylvin on AITC-induced acute inflammatory paw edema and IL-6 production. An intraplantar injection of the direct TRPA1 opener allyl isothiocyanate (AITC, 50 µg) induced an acute inflammatory paw edema and that was inhibitable by treatment with pinosylvin (10 mg/kg), resveratrol (10 mg/kg) or the TRPA1 blocker TCS 5861528 (10 mg/kg) (A). Correspondingly, the production of the proinflammatory cytokine IL-6 into the inflamed subcutaneous paw tissue injected with AITC was attenuated after treatment with pinosylvin, resveratrol or TCS 5861528 (B). Drugs were given intraperitoneally 1 h prior to the AITC injection and the edema was measured with a plethysmometer before and up to 6 h after the AITC injection and compared to the basal level. The inflamed subcutaneous paw tissue and the paw injected with the solvent from the vehicle treated mice (control) were dissected and the concentrations of IL-6 were analysed by ELISA. The results are expressed as mean + SEM, n=6-8, *=p<0.05, ***=p<0.001. Modified from Moilanen et al. Basic Clin Pharmacol Toxicol, 2016; 118(3): 238-242.

4.6 Final Summary of the Results

These studies focused on the role of TRPA1 in various aspects of acute inflammation and finally aimed at identifying pinosylvin as a novel TRPA1 blocker both *in vitro* and *in vivo*. The results were coherent and propose TRPA1 to possess a significant proinflammatory role in different types of experimental inflammatory diseases. Based on our preclinical findings, TRPA1 activation could be associated with diseases such as acute gout flare, the inflammatory component of osteoarthritis and acute allergic inflammation. Furthermore, TRPA1 activation was found to mediate carrageenan-induced paw edema and the direct activation of TRPA1 was inhibited by pinosylvin.

In all of the conducted studies, the function of TRPA1 was highly associated with the formation of acute edema. Curiously, the effects were not limited to the inflammatory edema as the findings indicated that in many cases, TRPA1 inhibition was effective in reducing the production of proinflammatory mediators and was even sufficient to reduce the symptoms and histopathological changes in MIA-induced experimental osteoarthritis. At least in the models of MIA-induced and ovalbumin-induced acute inflammatory edema, the effects of TRPA1 are likely mediated by the release of neuropeptides, especially substance P. This is supported by the findings that the treatment with L703,606, the blocker of the target receptor of substance P, was approximately as effective as the TRPA1 blocker and furthermore, the inhibition of TRPA1 resulted in a reduced release of substance P to the site of inflammation.

Interestingly, the stimulants used to trigger inflammation, i.e. carrageenan, MSU crystals, MIA and ovalbumin, did not activate TRPA1 directly. Previously the role of TRPA1 in inflammation has been regarded mainly as an initial direct sensor for exogenous irritating/proinflammatory compounds. However, based on our findings, TRPA1 is likely to play a role in the immune system as an endogenous factor, enhancing the inflammation initiated by a variety of mechanisms. This is a rather new perspective to understanding the function of TRPA1 and it may well pave the way to considering TRPA1 as a new drug target for treating many inflammatory diseases.

5 Discussion

5.1 Methodology

5.1.1 Animal Testing

All of the studies in this thesis included animal work performed in mice. When compared to *in vitro* experiments, one obvious advantage of using animal models is the possibility to study effects at the scale of a tissue and even the whole organism. For example, edema formation requires the interplay to occur between a properly functioning vasculature, the nervous system and tissue resident cells i.e. the reaction is complex and involves appropriately timed and finely-tuned inputs from different cell types and systems. In addition, the use of animal models gave us an opportunity to study behavioural nociceptive responses in the weight-bearing test which would be impossible to evaluate *in vitro* or by other alternative methods.

Disadvantages in the animal models lay in the limitations to the standardization of the experiments and the resources required for high quality research. Even though the animals were housed under standard conditions and the results were always compared within the same mouse strain matched as far as possible according to age and sex, there is always the presence of variation. The mice are individuals and this inevitably leads to variations within the studied groups. Many other external sources may exert an impact on the experiment results: the time of the day or day of the week, stress levels of the mice, experimenter associated factors or equipment related factors. In addition to the important ethical consideration which will be discussed in the next paragraph, animal testing requires a great deal of time and financial resources which limit the possibility of performing multiple study settings with multiple doses, time points and large groups. Therefore, based on the literature and previous experience, we always aimed at optimal timing and dosing with the minimal needed number of animals.

Perhaps most importantly, ethical consideration must be taken into account while conducting animal experiments. The performance of unnecessary animal tests, excess usage of animals and inhumane testing conditions is never ethically

acceptable. One of the earliest guidelines for animal studies, The Three R's Principle (Replacement, Reduction, and Refinement), was described by Russel and Burch in 1959 (Russell & Burch, 1959). Nowadays, the animal testing is strictly regulated by national laws (in Finland the law 497/2013) and in Europe, by the Directive on the protection of animals used for scientific purposes (2010/63/EU). All of our animal tests were carried out in accordance with these laws and directives and all of the experiments were approved by The National Animal Experiment Board. Proper attention was devoted to the welfare of the animals and the mice were provided with sufficient anaesthesia during the experiments and euthanized immediately afterwards.

At present, two distinct mouse strains carrying a deficient trpa1 gene have been developed. In 2006, Kwan and co-workers presented their TRPA1 deficient mouse strain. The mice were initially described with an unresponsive TRPA1 ion channel and attenuated nociceptive behaviour (Kwan et al., 2006). The mice are mixed BL6;129P –strain and the gene encoding TRPA1 has been made dysfunctional by disrupting the S5 and S6 transmembrane domains containing the pore-loop encoded by exons 22, 23 and 24. Another TRPA1 deficient mouse strain was also developed in 2006 by Diana Bautista and co-workers. This strain is bred in the C57BL/6 –mouse strain and the function of TRPA1 is prevented by deleting only the pore-loop encoded by exon 23. These transgenic mice also exhibit attenuated nociception and hypersensitivity (Bautista et al., 2006). Both of these TRPA1 deficient mouse strains have been studied widely and have contributed largely to our knowledge of the function of TRPA1. In study I, the TRPA1 deficient mice originated from Diana Bautista and David Julius and were back-crossed to C57BL/6-strain due to their chimeric nature by our Swedish colleagues Peter Zygmunt and Edward Högestätt. In later studies II-V, the commercially available mixed BL6;129P –strain was used in the TRPA1 deficient murine experiments. In the control experiments, the two different TRPA1 deficient mouse strains acted similarly in the carrageenan-induced acute inflammatory paw edema test.

The tremendous advantage of genetic silencing of the gene of interest, i.e. TRPA1 in this case, is the certainty of full dysfunctionalization of the wanted target. In addition, the avoidance of off-target effects which are almost unavoidable in experimental drug treatment *in vivo* is secured by utilizing genetically deficient animals. In cell culture, a corresponding degree of specificity can be achieved by adopting the siRNA approach. However, there are some restrictions in the data obtained in genetically depleted animals. Firstly, the lack of a certain gene may affect

the development of the organism. Secondly, there is the possibility of the development of compensatory mechanisms and the disruption of interactions with other channels, such as TRPV1 (Patil et al., 2010), should be remembered. It should also be noted that although mice are mammals, they are somewhat different from humans and therefore any extrapolation of results to human diseases should be made with caution. The fact that human and rodent TRPA1 have only 79% sequence homology should also be noted during the interpretation of the murine data (Chen & Kym, 2009; Chen et al., 2013). Nevertheless, the validity of our results obtained from TRPA1 deficient animals was supported by the fact that treatment with two distinct TRPA1 blockers, HC-030031 and TCS 5861528, produced extremely similar results.

5.1.2 Acute Inflammation Edema Test

The most widely used animal test in this thesis was the model of acute inflammatory paw edema. The edema was induced by intraplantar injection of a variety of stimulants: carrageenan, AITC, MSU crystals, MIA and ovalbumin. The effect of external unknown contaminants was minimized by using sterile equipment and solutions and by disinfecting the injection site with ethanol. The severity of the edema was measured by measuring the paw volume and comparing it to the baseline volume measured prior to the injection. The border of the measured area was carefully marked on the paw to minimize variation during the repeated measurements. The repeated measurements conducted during a single experiment were also performed by the same investigator to avoid variation. The volume of the injection was kept small and the contralateral paw injected with the vehicle did not display any measurable edema. The studied mice were anesthetized during the whole experiment primarily to reduce the distress to the animals but also to minimize the effect of pain and stress on the development of edema.

The accumulation of the studied proinflammatory factors (substance P, IL-4 and IL-6) was measured after the paw edema test. The tissue was extracted by dissecting the specific plantar area of the paw where the initial injection had been made. However, performing an ultimately standardized dissection of soft tissue is extremely difficult and therefore there is a possibility of variation within the injected group. Next, the tissue was broken down chemically using established protocols (Bilici et al., 2002; B. Liu et al., 2013) and analysed for cytokine contents by ELISA. The contralateral paw injected with the vehicle was also analysed and only low cytokine

concentrations were detected as compared to the paw injected with the stimulant. This was a sign that the increase in cytokine content was indeed due to the injection of the stimulant.

5.1.3 Weight-Bearing Test and Histopathological Changes in Experimental Osteoarthritis

In the weight-bearing test, the nociceptive behaviour indicative of joint pain was analysed by using purpose-built special scale, i.e. an incapacitance meter. The scale has two separate pressure sensors; one for each hind limb and a distinct non-scale slope on which the front limbs can rest. The strength of this test is in the fact that the mice freely distribute their weight between their hind limbs and no external putative pain causing stimuli is applied; thus the test assesses spontaneous pain or allodynia. However, especially in the MIA-induced experimental osteoarthritis (study III), the changes observed were relatively small in magnitude although in line with previous results with this model (Ogbonna et al., 2013). This was probably due to the use of mice as the test animals as their responses are smaller than encountered in rats, which are used more frequently. Error sources were minimized by injecting the contralateral joint with the vehicle to reduce the effect of the injection. Furthermore, eight repeated measures were performed at each time point and the investigator of the weight distribution was blinded to which was the affected limb. In study III, behavioural testing was associated with histopathological analysis. This indicates that the nociceptive behaviour induced by MIA-injection is not only due to sensitization and most likely related to cartilage changes as the histopathological changes were similarly more prominent in the wild type mice than in their TRPA1 deficient counterparts. If one wished to obtain a conclusive correlation between the weight-bearing findings and nociception then further testing would be needed, such as electrophysiological recordings in the innervating sensory nerves.

5.1.4 Air-Pouch Inflammation

The murine joint cavities are extremely small and hence the collection of synovial fluid samples would be an extremely difficult task, if not impossible. As an alternative method to model synovial joint arthritis, we used the air-pouch inflammation test. This is a validated method in which a joint resembling cavity is created by injecting sterile air subcutaneously beneath the mouse skin. Previous studies have shown that

during a one-week period, the cavity develops a synovium-like inner layer (Edwards et al., 1981; Forrest et al., 1988). In detail, the two lining cell types are macrophages and fibroblasts which closely resemble the type A and B cells of the actual synovial membrane (Edwards et al., 1981). Hence, the air-pouch provides a good platform from which to study experimental arthritis as it is readily accessible. In our experiments, the danger of contamination derived infection was reduced by performing a careful purification of the injection site, by injecting sterile air to form the air-pouch and by using endotoxin-free MSU crystals and solutions and sterile equipment for injections. After the experiments, the air-pouches were rinsed and the collected fluids were analysed for cell count with a hemocytometer and for cytokine concentrations by ELISA according to standard procedures.

5.1.5 Ca^{2+} Current Measurements and Cell Culture Experiments

The Fluo-3-AM assay used in this thesis for measurement of Ca^{2+} influx in TRPA1 expressing HEK 293 cells is a widely exploited method. The TRPA1 expression was induced by transient transfection with the hTRPA1 vector to obtain stable and high expression of the ion channel. Next, the cells were exposed to the known TRPA1 opener AITC or to a test compound. As nontransfected cells did not exhibit Ca^{2+} influx in response to AITC, the effect was regarded as being specific for TRPA1. This was further supported by the findings with the treatment with HC-030031, which was used as a control compound because it has been used widely and regarded as a specific TRPA1 blocker (Eid et al., 2008). The blocking properties of the studied compounds were assayed by adding the compound to the cells 30 min before the application of AITC. These methods are frequently used and therefore the results obtained can be regarded as reliable. However, the antagonistic properties of pinosylvin were studied additionally in patch clamp measurements. In these studies, pinosylvin was able to inhibit the membrane current induced by AITC even when pinosylvin was applied after the AITC. As similar results demonstrating the ability of pinosylvin to inhibit TRPA1-mediated currents were obtained using two distinct methods, the finding can be regarded as very reliable. It should be noted that the Fluo-3-AM assay and the patch clamp measurements were performed at room temperature. Theoretically, temperature might influence the ion channel sensitivity and this should be kept in mind in the interpretation of data and extrapolations made from the results to settings in physiological temperatures.

The findings in animal models were complemented with *in vitro* experiments. HEK 293 cells transfected with TRPA1 (study I), human primary osteoarthritic chondrocytes (study III) and murine cartilage tissue (study III) were cultured. The expression of inducible inflammatory gene COX-2 was analysed after exposing the cells to AITC or to MIA and IL-1β and the cells were treated with the TRPA1 blocker to identify the role of TRPA1 activation. In addition, TRPA1 deficient murine cartilage was examined. The strength of cell culture experiments is in the standardized experiment environment and the possibility of exploring more detailed effects. However, the cell culture lacks many of the physiological features of tissues, such as interactions with other cell types, and therefore the interpretation and relevance of the findings to the actual diseases might be challenging.

5.2 TRPA1 Activation Enhances Inflammation

The principal hypothesis in this series of studies making up this doctoral thesis was that TRPA1 activation would play a major role in the development of various types of acute inflammation and the findings largely supported this hypothesis. In summary, in the first study, we found that TRPA1 activation mediated the formation of carrageenan-induced acute inflammatory paw edema, which is a widely used model of acute inflammation. The second study produced results in which TRPA1 activation was crucial for MSU crystal-induced experimental gout in acute paw edema, air-pouch inflammation and weight-bearing test. Further, in the third study, we observed that MIA-induced acute inflammation *in vivo* and *in vitro* was TRPA1-dependent and that functional TRPA1 was important for the development of experimental osteoarthritis. The fourth study focused on the experimental ovalbumin-induced allergic inflammation and the results showed that TRPA1 was highly involved in the development of allergic paw inflammation and conjunctivitis without interfering with the actual sensitization process. Finally, the last study revealed the potency of pinosylvin to inhibit TRPA1 *in vitro* and *in vivo*.

5.2.1 Previous Knowledge on TRPA1 in Inflammation

Originally, the function of TRPA1 was discovered in neurons and the activation of TRPA1 was found to mediate nociceptive sensations induced by exogenous irritating compounds (Bandell et al., 2004; Jordt et al., 2004; Story et al., 2003). Thereafter, exogenous and direct TRPA1 activations were observed to enhance inflammatory

pain and hypersensitivity (Bautista et al., 2006; Kwan et al., 2006). Long before the identification of TRPA1, AITC had already been used as a stimulant to induce neurogenic inflammation and it was known to be dependent on the release of proinflammatory neuropeptides such as CGRP, substance P and neurokinin A (Bánvölgyi et al., 2004; Inoue et al., 1997; Lynn & Shakhanbeh, 1988). Subsequent studies indicated that TRPA1 activation induced the release of neuropeptides CGRP and substance P followed by an arterial dilatation and plasma extravasation (Bautista et al., 2005; Trevisani et al., 2007). A further advance in knowledge was made as endogenous TRPA1 channel openers were identified and most of them seem to be included in the "inflammatory soup". Namely, ROS and RNS and their reactive metabolites such as 4-hydroxynonenal, 4-hydroxyhexenal, 4-oxononenal and nitrooleic acid have been identified as activating TRPA1 (Andersson et al., 2008; Taylor-Clark et al., 2009; Trevisani et al., 2007). In addition to direct activation, TRPA1 has been shown to be susceptible to upregulation and/or sensitization by many proinflammatory factors such as bradykinin, trypsin, IL-6, hypoxia-inducible factor-1α and nerve growth factor (Bandell et al., 2004; Dai et al., 2007; Hatano et al., 2012; Malsch et al., 2014; Obata et al., 2005)

Simultaneously with our studies, other evidence has been gathering on the proinflammatory role of TRPA1. Experiments conducted with a TRPA1 blocker or genetic disruption of the ion channel have shown that TRPA1 is crucial in experimental inflammatory models mimicking colitis, allergic asthma, non-allergic airway hyperreactivity, atopic dermatitis and gout flare (Caceres et al., 2009; Engel et al., 2011; Hox et al., 2013; B. Liu et al., 2013; Trevisan, Materazzi et al., 2013). Most of these studies have focused on TRPA1 in inflammatory conditions, revealing that TRPA1 is indisputedly involved in pain and hypersensitivity (Andrade et al., 2012). There is also evidence that direct TRPA1 activation induces plasma extravasation, mast cell degranulation, prostaglandin production and neutrophil migration (Perin-Martins et al., 2013). Furthermore, an interesting study conducted by evaluating the experimental dermatitis induced by oxalozone, a direct TRPA1 activator, showed that in addition to histopathological inflammation, the levels of many proinflammatory cytokines such as CXCL-2, IL-4, IL-6 and serotonin were decreased as a consequence of TRPA1 inhibition (B. Liu et al., 2013). In addition, an indirect activation of TRPA1 is putatively involved in the pathogenesis of experimental gout and asthma and TRPA1 stimulation has been shown to increase proinflammatory mechanisms including neutrophil migration, edema formation and production of cytokines such as IL-13, IL-5, TNF-α, MCP-1 and IL-1β (Caceres et al., 2009; Trevisan, Hoffmeister et al., 2013; Trevisan, Hoffmeister et al., 2014).

Hence, it is very likely that if the stimulant is unable to directly activate TRPA1, this is due to the endogenous production of TRPA1 activators such as ROS or RNS (B. Liu et al., 2013; Trevisan, Hoffmeister et al., 2014).

5.2.2 TRPA1 and Acute Inflammatory Edema

In our experiments, we were able to prove that proinflammatory stimulants including carrageenan, MSU crystals and MIA induced acute edema via a TRPA1-dependent mechanism. Furthermore, in sensitized mice, a subcutaneous challenge with the specific allergen ovalbumin induced an acute paw edema susceptible to TRPA1 antagonism. Another highly important finding was that none of these stimulants was a direct activator of TRPA1, indicating that TRPA1 activation is downstream in the cascade of events leading to the formation of acute inflammatory edema. Similar findings have been previously described for MSU and ovalbumin, which fail to stimulate murine TRPA1-mediated Ca^{2+} currents (Hox et al., 2013; Trevisan, Hoffmeister et al., 2013).

The background for edema development lies in the complex intra- and intercellular signalling that elicit the increase in blood flow and endothelium permeability, which in turn lead to plasma extravasation to the site. There are other adjacent cells such as dendritic cells, mast cells or neurons which release humoral inflammatory factors, like histamine, leukotrienes, and neuropeptides and these can affect the endothelial cells and activate them to contract and allow increased fluid and protein leakage from the vessel. This is supported by the increased blood flow promoted by vasodilation which is also induced by the effects of factors such as histamine, prostanoids (especially prostacyclin and prostaglandin E_2) and NO on vascular smooth muscle (Kumar et al., 2010). Based on the convincing evidence of the function of TRPA1 in sensory nerve endings, the most obvious explanation is that TRPA1 mediates its effects through the neuronal contribution in inflammation, i.e. neurogenic inflammation. In this hypothesis, an initial stimulus triggers the production of endogenous TRPA1 channel openers; these activate TRPA1 on the local sensory nerve ending and this activation then stimulates the release of proinflammatory neuropeptides and finally it is the proinflammatory peptides that exert vascular effects, resulting in the acute edema.

As discussed, many ROS, RNS and their reactive metabolites are potent endogenous TRPA1 activators and therefore they are likely to be key regulators in the development of TRPA1-induced acute inflammatory edema. In study III, we

were able to inhibit the MIA-induced acute edema not only with the TRPA1 blocker but also with the H_2O_2 degrading enzyme, catalase, i.e. the formation of H_2O_2 is a key regulator involved in the early events of MIA-induced inflammation. Interestingly, previous studies have also shown the ability of MIA to increase the production of ROS (Jiang et al., 2013). Similar findings have been made in the MSU crystal-induced inflammation and it is known that although they have many mechanisms of action, one important property of MSU crystals is their ability to increase ROS production and treatment with catalase has been reported to reduce the severity of experimental gout (Shi et al., 2010; Trevisan, Hoffmeister et al., 2013). Furthermore, the ROS, RNS and their reactive metabolites have been shown to be highly edematogenic in a TRPA1-dependent manner (Graepel et al., 2011; Trevisan, Rossato et al., 2014; Trevisani et al., 2007). Complementary to this, TRPA1 is also sensitized by secondary messengers phospholipase C and protein kinase A, which are known to be activated by many proinflammatory mediators through their G-protein coupled receptors (S. Wang et al., 2008).

The secondary effector mechanism subsequent to TRPA1 activation in the acute inflammatory edema is most likely the release of proinflammatory neuropeptides, especially substance P. Our results indicated that the blockade of the target of substance P, i.e. the NK1 receptor, by L703,606 effectively inhibited the development of the acute edema induced by MIA and ovalbumin. Furthermore, the analysis of substance P levels in the MIA-injected inflamed paw tissue demonstrated that treatment with the TRPA1 blocker TCS 5861528 was effective in decreasing the release of substance P. Our hypothesis was supported by the finding that treatment with L703,606 did not alter the levels of substance P. Similar results on the potency of NK1 receptor blockade on TRPA1-induced acute edema have been reported elsewhere (Silva et al., 2011), further supporting our findings.

In addition to the neuronal mechanism described above, the function of TRPA1 in endothelial cells warrants discussion. The role of endothelial cells in edema formation has been well documented and were TRPA1 to exert a role in the function of endothelial cell, this could provide an alternative mechanism for development of TRPA1-mediated edema. Indeed, the expression of TRPA1 in endothelial cells has been described and the direct activation of TRPA1 in these cells has been found to promote vasodilation (Earley et al., 2009). In addition, endothelial TRPA1 activation has also been shown to respond to ROS stimulation by vasodilation (Sullivan et al., 2015). The involvement of endothelial TRPA1 in edema, specifically in the acute inflammation, however remains unknown, but it is an interesting possibility.

Furthermore, both neurogenic TRPA1-induced release of proinflammatory neuropeptides and non-neurogenic TRPA1-mediated effects such as those in the endothelium may act simultaneously and contribute jointly to the formation of inflammatory edema.

5.2.3 TRPA1 and Production of Proinflammatory Factors

The intracellular concentration of Ca^{2+} has many effects including influencing the regulation of inflammatory genes (Berridge et al., 2000; Jakobsson, 2010; Korhonen et al., 2001). This provides a foundation for the speculation that since TRPA1 activation can increase the intracellular Ca^{2+} concentration, it could also promote the production of proinflammatory factors. Indeed, our findings of a proinflammatory role for TRPA1 was not limited to the acute extravasation and pain. On many occasions, the production of proinflammatory factors was substantially attenuated after the inhibition of TRPA1 proposing that the effects of TRPA1 activation extend to more profound aspects of the inflammatory cascade and are not simply restricted to the release of vasoactive neuropeptides. We found that the production of the allergy associated early cytokine, IL-4, in the allergic ovalbumin-induced inflammation and the production of IL-6 in the AITC-induced paw inflammation were blunted after treatment with a TRPA1 blocker. In addition, the experiments on the MSU crystal-induced gouty inflammation in the air-pouch also revealed that TRPA1 activation was crucial for the accumulation of a plethora of cytokines including MCP-1, IL-6, IL-1β, MPO, MIP-1α and MIP-2. In addition, the accumulation of cells, thought to be mainly polymorphonuclear leukocytes, was diminished in the mice treated with the TRPA1 blocker as was also the case in animals lacking a functional TRPA1 channel. Furthermore, the accumulation of eosinophils in the late-phase of the acute allergic inflammation was attenuated after treatment with the TRPA1 blocker in the ovalbumin-induced conjunctivitis model. Most of the measured cytokines are not stored intracellularly in great quantities and their production is regulated by alterations of gene expression. Hence, it is likely that TRPA1 activation, directly in the same cells or indirectly through intercellular signalling, has an impact on the transcriptional or posttranscriptional regulation of several inflammatory genes.

In addition to the direct regulation of gene expression, other mechanisms also contribute to the increased production of proinflammatory factors. The elevation of the intracellular Ca^{2+} level has been shown to increase the activity of some

proinflammatory enzymes such as polymerase-1 which is required for the nuclear translocation of the NF-κB transcription complex and calmodulin-dependent kinases (Racioppi & Means, 2008; Vuong et al., 2015). Furthermore, elevation of the intracellular Ca^{2+} concentration is crucial for the release of factors stored in endosomes, i.e. exocytosis. For example, this is an important mechanism in release of histamine and serotonin from mast cells. Since the activation of TRPA1 induces a direct Ca^{2+} influx leading to an elevation of the Ca^{2+} concentration, it is plausible that TRPA1 is involved in these processes.

Interestingly, ion channels can carry out their secondary effects through diverse mechanisms. Obviously, the flux of ions leading to the changes in intracellular ion concentrations and altered voltage gradients is, by definition, a significant mechanism of action. However, there are data showing that some ion channels also contribute to modulate cellular behavior through additional mechanisms mimicking receptor-mediated events. For instance, the L-type calcium channel has been demonstrated to regulate the activity of the transcription factor, nuclear factor of activated T-cells (NF-AT) which is recognized as being involved in immune responses. Importantly, this effect is independent of the Ca^{2+} concentration and opening of the ion channel but is mediated via the activation of the C-terminus of the ion channel (Kudryavtseva et al., 2013). Since it is known that TRPA1 also has a large intracellular C-terminal moiety, it is plausible that TRPA1 activation exerts also Ca^{2+}-independent effects which could contribute to the observed changes, such as an increase in the production of the proinflammatory cytokines.

We and other research groups have shown that TRPA1 activation promotes vascular effects leading to extravasation (Zygmunt & Högestätt, 2014). One clear effect is the enhanced vascular permeability which not only induces an increase of fluid volume in the tissue, but also an exudation of many plasma-derived proteins and other factors. This exudate contains the components of four inflammation-associated proteolytic enzyme cascades, namely the complement, coagulation, fibrinolytic and kinin systems which may become activated in the inflamed tissue to generate a wide range of proinflammatory and regulatory factors, e.g. the activated complement components C3a and C5a as well as bradykinin which augment the inflammatory response by enhancing the production of prostaglandins and other inflammatory mediators and/or by promoting the chemotaxis of leukocytes to the site of inflammation. Increased vascular permeability is also associated with the activation of endothelium, leading to further leukocyte migration into the inflamed tissue. In the present study, antagonism of TRPA1 caused a down-regulated

extravasation and less leukocyte accumulation within the tissue. Interestingly, those effects could also indirectly have contributed to the observed reduction of the levels of inflammatory mediators in TRPA1 blocker treated and TRPA1 deficient animal tissue samples because abundant cytokine and eicosanoid production is a characteristic feature of activated leukocytes. (Kumar et al., 2010; Rang et al., 2015b)

It is interesting, that even though the initial triggers of inflammation were all very different, the anti-inflammatory potential of inhibition of TRPA1 was clear in all the experiments. In fact, since these findings were rather novel, they can be considered as opening a new perspective for the role of TRPA1 in acute inflammation and there are some very recent findings which support our results. In a model of MSU crystal-induced gouty arthritis published just prior to our results, it was noted that MSU crystal-induced cytokine production was TRPA1-dependent (Trevisan, Hoffmeister et al., 2014). In addition, there is also *in vitro* evidence of a TRPA1 activation-induced increase in the production of IL-1β, IL-6, IL-8 and prostaglandin E_2 in keratinocytes, skin fibroblasts and periodontal ligament cells (Atoyan et al., 2009; Jain et al., 2011; Son et al., 2015). In summary, these results emphasize that the role of TRPA1 activation in inflammation is not limited to pain, hypersensitivity and extravasation but impacts also on other aspects of the inflammatory cascade and these effects are mediated by distinct mechanisms that will need to be clarified in the future.

5.2.4 Association of TRPA1 and COX

There was an interesting interaction between the prostaglandin producing COX enzyme and TRPA1. Firstly, we found that the COX inhibitor, ibuprofen, was effective in attenuating the carrageenan- and AITC-induced paw edema, which were also clearly TRPA1-dependent effects. This result does not clearly represent the temporal sequence of events, but as AITC's mechanism of action is the direct activation of TRPA1, the activation of prostaglandin production should occur after the TRPA1 stimulation. The carrageenan-induced acute inflammatory edema has been shown to be clearly COX-dependent (Morris, 2003). As described earlier, the TRPA1 activation induces the release of prostaglandin E_2, which is a potent proinflammatory eicosanoid (Jain et al., 2011). As the isoform mainly responsible for the inflammatory production of prostaglandins, COX-2, is highly inducible, we decided to clarify whether TRPA1 activation would upregulate COX-2 expression *in vitro*. Indeed, in HEK 293 cells transfected with TRPA1, application of the direct

TRPA1 activator, AITC, enhanced COX-2 expression, a phenomenon not observed in nontransfected HEK 293 cells.

Furthermore, an indirect TRPA1 activation resulting from exposure to MIA and IL-1β was able to upregulate COX-2 expression in human primary osteoarthritic chondrocytes and also in murine cartilage and this effect was reversed with TRPA1 antagonism or genetic knock-out. In chondrocytes and cartilage, the observed effect was most likely mediated via further autocrine or intracellular regulation as there was no direct MIA-induced TRPA1 activation. In addition, there are reports showing that the reactive metabolites of prostaglandin E_2 including prostaglandin A_2, A_1 and J_2 have been identified to activate TRPA1 giving rise to a hypothetical feed-forward mechanism (Taylor-Clark et al., 2008). According to our findings, the TRPA1-dependent COX-2 upregulation noted after exposure to MIA was linked to IL-1β co-stimulation. This effect cannot be conclusively explained by our results but previous reports have shown that IL-1β is capable of increasing the TRPA1 expression (Hatano et al., 2012). The concept of TRPA1 being an inducible proinflammatory regulator having an impact also on other inducible inflammatory genes is very interesting, but further studies will be needed to clarify the magnitude and significance of this mechanism.

The findings of COX-2 upregulation were made in chondrocytes and HEK 293 cells, highlighting the role of non-neuronal TRPA1. Much of the research on TRPA1 has concentrated on neurogenic inflammation and the function of TRPA1 in neurons. As stated in the Review of the Literature, TRPA1 has been identified and found functional in many non-neuronal cells (Fernandes et al., 2012). Some of the most interesting findings include the role of TRPA1 in endothelial cells, keratinocytes, fibroblasts and mast cells, which will be discussed later. Studies in skin keratinocytes and lung fibroblasts have identified that TRPA1 acts as a proinflammatory factor also in these tissues. Subsequent to its activation, a TRPA1-dependent increase in IL-1 and IL-8 was observed (Atoyan et al., 2009; Mukhopadhyay et al., 2011). Our experiments add chondrocytes to the list of non-neuronal cells expressing TRPA1 and its activation seems to upregulate the expression of COX-2 in these cells. The proinflammatory role of TRPA1 in non-neuronal cells is very interesting and may well open up new treatment strategies for many inflammatory diseases. However, this does not negate the function of neuronal TRPA1 but rather supplements it. The possible interaction of different cell types regarding the activation of TRPA1 leave many questions unanswered i.e. the targeted

TRPA1 ion channel and the production of the endogenous TRPA1 activators may not necessarily be the same cell types.

5.2.5 TRPA1 Activation Enhances Pain and Inflammation in Arthritis

Two distinct arthritis models were exploited in the thesis, MSU crystal-induced acute gouty arthritis (study II) and MIA-induced experimental osteoarthritis (study III). The studied mice were tested for their nociceptive behaviour in the weight-bearing test. In gouty arthritis, the effect was fairly acute and the mice displayed a shifting of their weight away from the affected limb already one day after the injection of MSU crystals, which reflects the clinical characteristics of the rapid onset of gout flare. In MIA-induced experimental osteoarthritis, the response developed more slowly over a few weeks, after which the histopathological changes were also observed. The observed joint pain was obviously due to the inflammation in the affected area, leading to activation of nociceptors. In addition to mechanical and thermal factors, many local substances of the "inflammatory soup" such as protons, ATP, bradykinin and prostaglandins act to excite the nociceptor to reach its firing potential to produce painful sensation. Numerous target receptors on the peripheral nerve ending have been shown to be involved in pain sensing (Rang et al., 2015a). Interestingly, peripheral targeting of TRPA1 has been shown to be effective in alleviating especially acute pain induced by stimulants such as carrageenan, Freund's complete adjuvant or MSU crystals (Bonet et al., 2013; Fernandes et al., 2011; Trevisan, Hoffmeister et al., 2014).

Pain associated with inflammation involves also central mechanisms as discussed earlier in the Review of the Literature section. However, central (i.e. intrathecal) administration of TRPA1 blocker has failed to attenuate the acute pain experienced almost immediately after peripherally applied formalin (Wei et al., 2011). However, with a longer follow-up, the central inhibition of TRPA1 has been shown to alleviate the delayed hypersensitivity or pain lasting for days to weeks after the initial peripheral stimulus, indicating that central TRPA1 could play a role in the process of central pain sensitizing (da Costa et al., 2010; Wei, Koivisto et al., 2010). It should be noted that central pain sensitization has been reported as a nociceptive mechanism in MIA-induced experimental osteoarthritis (Ferreira-Gomes et al., 2012; Kelly et al., 2012; Y. Lee et al., 2011). Furthermore, a single dose of the TRPA1 blocker, given either systemically or intra-articularly, is ineffective in attenuating the nociceptive behaviour measured by weight-bearing test immediately or several days

after the injection of MIA into the joint (Okun et al., 2012). Nonetheless, we observed that the TRPA1 deficient mice displayed an attenuated nociceptive behaviour in response to MIA a few weeks after the injection. In summary, it seems likely that TRPA1 has a role in both peripheral activation of nociceptors and central pain sensitization. Further studies will be needed to be able to determine the proportion of contribution of these mechanisms in pain and hypersensitivity in different modalities and stages of arthritis.

5.2.6 Allergic Inflammation and TRPA1

In the fourth study, we explored the relationship between TRPA1 activation and acute allergic inflammation. The results indicated that TRPA1 activation was essential for the early-phase of the reaction as evidenced by an attenuated paw edema present within one hour after the allergen challenge with treatment with TRPA1 blocker or by genetic silencing of TRPA1. Correspondingly, the development of the allergic inflammation was dampened as reflected by the reduced level of IL-4 at 6 h after the ovalbumin application. The late-phase of the acute allergic inflammation was also alleviated as the accumulation of eosinophils into the inflamed conjunctival tissue could be decreased by blockade of TRPA1, achieved either pharmacologically or genetically. Interestingly, the sensitization towards ovalbumin remained intact. These results are supported by previous findings demonstrating the proinflammatory role of TRPA1 in ovalbumin-induced allergic asthma (Caceres et al., 2009; Wu et al., 2013) and the development of IL-13-induced itch (Oh et al., 2013). In addition oxalozone, a compound frequently used to induce experimental atopic dermatitis, is also a direct activator of TRPA1 (B. Liu et al., 2013). Similarly, another previously known contact sensitizer, 2,4-dinitrochlorobenzene, has proven to be a direct TRPA1 activator (Saarnilehto et al., 2014).

Apart from our experimental settings, the previous studies on ovalbumin-induced respiratory inflammation analysed the outcome of a repeated or continuous exposure lasting from many days to weeks. There was also no evidence of direct activation of TRPA1 by ovalbumin or disturbed sensitization towards ovalbumin as reflected by unaltered levels of circulating ovalbumin specific IgE (Caceres et al., 2009; Wu et al., 2013). These published papers have results parallel to those observed here i.e. the response in splenocytes to ovalbumin was similar in sensitized TRPA1 deficient and corresponding wild type mice. In addition to the neurogenic mechanisms proposed

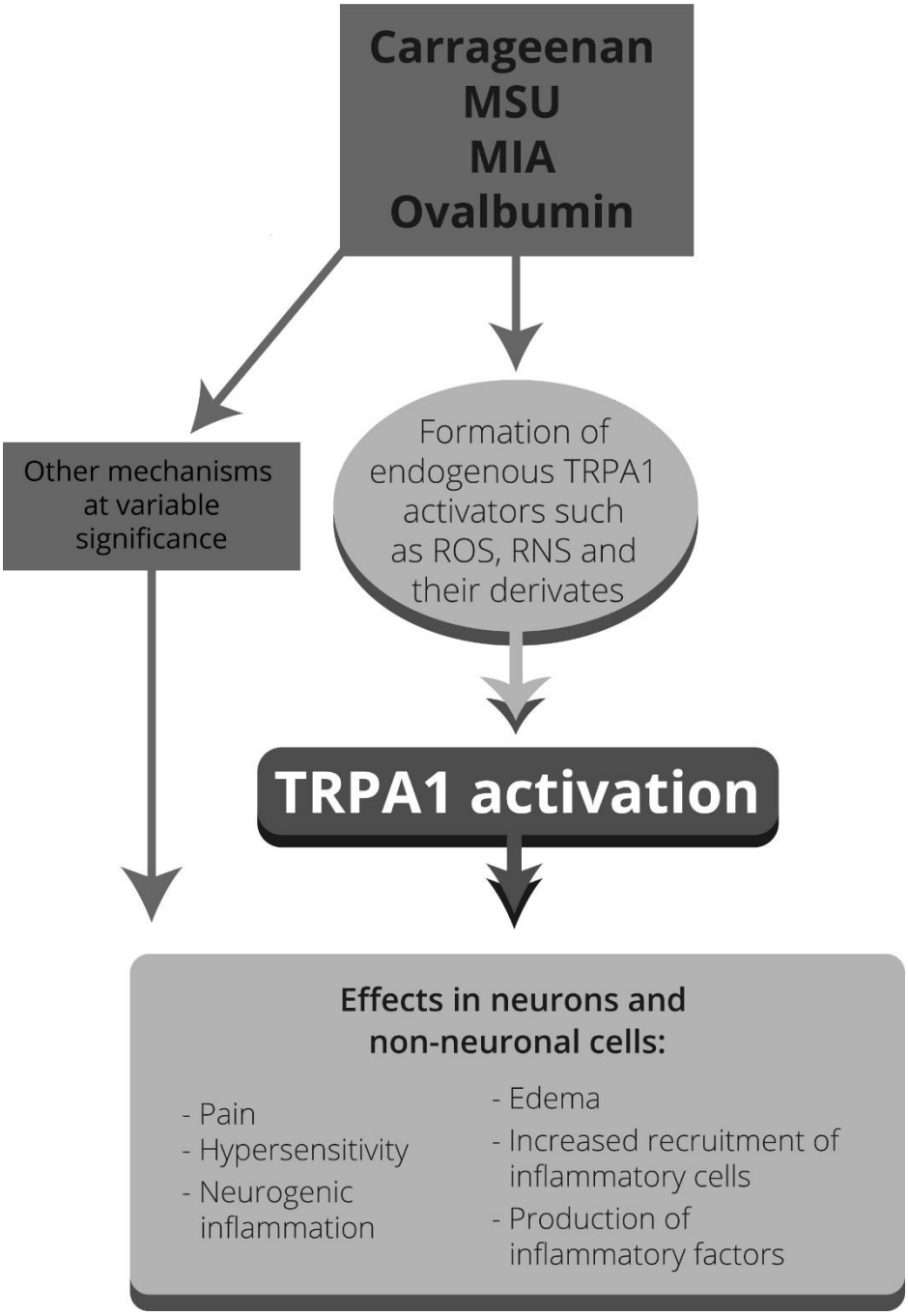


Figure 22. Schematic summary of the role of TRPA1 activation in inflammation on the basis of studies I-IV. Contents design by Lauri Moilanen and graphical design by Joonas Mykkänen.

by the decreased levels of proinflammatory neuropeptides measured in the brochoalveolar lavage fluid after ovalbumin challenge (Caceres et al., 2009) and the efficancy of blockade of the receptor of substance P we discovered, previous studies have made interesting findings in mast cells. Firstly, TRPA1-mediatedproinflammatory effects can be alleviated by inhibiting mast cell degranulation (Perin-Martins et al., 2013). Furthermore, the expression and function of TRPA1 in mast cells have been detected (Oh et al., 2013; Prasad et al., 2008). Interestingly, TRPA1 expression is also upregulated by many factors such as IL-13 and leukotriene B_4 which are both characteristic mediators of allergic inflammation (Fernandes et al., 2013; Oh et al., 2013). Since degranulation occurring after mast cell activation is a crucial event in the very early events of allergic inflammation, it is possible that if TRPA1 were to be blocked, the degranulation could at least be partly inhibited and this could have an impact on the further development of the allergic inflammation.

The total amount of tissue infiltrated eosinophils is an indicator of the late-phase of the acute allergic inflammation process and our results showed that TRPA1 blockade, attained by either pharmacological or genetic means, could alleviate this process. According to the present results, the mechanism of TRPA1 activation does not involve any increase of eosinophil apoptosis which is at least partly the mechanism of action of the anti-inflammatory glucocorticoids. However, our results indicate that TRPA1 functions in the very early events of acute inflammation such as extravasation and in the production of IL-4 and inhibition and these effects could explain the TRPA1 inhibition-mediated alleviation of the late-phase acute allergic inflammation. The underlying mechanisms could involve either mast cells or innervating neurons, or both.

5.2.7 Pinosylvin is a Novel Blocker of TRPA1

Stilbenoids are a group of polyphenols found in natural sources such as in the heartwood and bark of the *Pinus* coniferous tree species. In an attempt to find novel TRPA1 blockers, we became interested in stilbenoids. This was based on our preliminary screening results and on previous findings indicating that another member of the stilbenoid group, namely resveratrol, was capable of inhibiting TRPA1 in addition to its other anti-inflammatory properties (Kulkarni & Canto, 2015; Yu et al., 2013). In fact, recent studies have also revealed that yet another

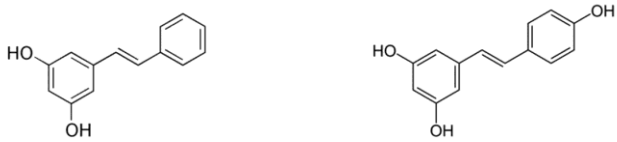


Figure 23. The chemical structures of stilbenoids pinosylvin and resveratrol. Chemically stilbenoids are classified as hydroxylated derivatives of stilbene, which is structurally composed of two phenyl rings joined by an ethane group.

polyphenol, gallic acid, displays potency at inhibiting TRPA1 (Trevisan, Rossato et al., 2014). In our experiments, one particular stilbenoid, pinosylvin, was found to inhibit TRPA1 in two different Ca^{2+} current assays. The effect was dose-dependent and pinosylvin was able to inhibit the effect of AITC when applied either prior or subsequent to AITC and it exhibited a fast onset and sustained duration of action. Similarly to resveratrol, the exact molecular mechanism to explain how pinosylvin inhibits the activation of TRPA1 remains unknown (Yu et al., 2013).

Suprisingly, pinosylvin and resveratrol were able to induce minor TRPA1-mediated currents in the patch clamp studies, raising the question if stilbenoids act as modulators rather than pure blockers of the TRPA1 ion channel. This stimulatory effect was observed only at high concentrations, far exceeding the IC_{50} values of the compounds. This kind of bimodal action on TRPA1 is not however restricted to stilbenoid based compounds. Some oxime derivate TRPA1 blockers, closely related to AP18 described earlier, have been shown to have a similar bimodal effect on TRPA1; they prevent the activation of TRPA1 dose-dependently but at high concentration they also activate the channel (Defalco et al., 2010). In addition, a reversed bimodal effect on TRPA1 has been discovered in some TRPA1 ligands. Compounds such as cinnamaldehyde, camphor and apomorphine act as TRPA1 activators at low concentrations but inhibit it at higher concentrations (Alpizar et al., 2013; Schulze et al., 2013). However, the molecular mechanism of this minor activation potential of stilbenoids remains a mystery. Classical explanations such as configurational isomerism or complementary sites of molecular interaction at high concentrations could explain the observed effects but more detailed studies will be needed to understand fully the pharmacological effects of the stilbenoids on TRPA1.

Further experiments showed that in experimental animals, systemic dosing with pinosylvin inhibited AITC-induced acute paw edema and the production of IL-6 within the inflamed paw tissue. This is very important as it highlights the potential of pinosylvin to inhibit TRPA1 functionally in a living animal. As pinosylvin seems to be a potential TRPA1 blocker *in vitro* and *in vivo*, the stilbenoids could be a source for future drug development of TRPA1 blockers.

The safety issues of new drug candidates should be kept in mind already during the early-phases of the drug development. Due to multiple targets of action, the adverse effects of stilbenoids are not easy to predict, although resveratrol has shown a favourable safety profile. However, if a high selectivity for TRPA1 could be achieved, the predictability of safety would increase. As already discussed, tests conducted in murine models have shown selective TRPA1 blockers to be relatively safe (McNamara et al., 2007). The main concern around the safety of TRPA1 blockers is the possibility of an impaired response to exogenous harmful compounds such as toxic gases (Bautista et al., 2006; Brone et al., 2008). Furthermore, TRPA1 is activated by many flavour- and scent-related compounds such as mustard oil and cinnamaldehyde and thereby inhibition of TRPA1 could alter the senses of taste or smell. The results from the ongoing clinical trials on TRPA1 inhibiting drugs are still pending and the information of these studies will provide very interesting and important data on the safety of TRPA1 blockers.

5.3 Future Prospects

The present results suggest that TRPA1 activation could be an important mechanism involved in many inflammatory diseases. We obtained positive results after treating mice with a TRPA1 blocker in experimental models of gout, allergic inflammation and osteoarthritis. TRPA1 activation seemed also to be important in the development of a more general model of acute inflammation, i.e. carrageenan-induced acute paw edema. Interestingly, the findings were not just limited to symptoms such as edema and pain, but antagonism of TRPA1 was also effective in inhibiting the underlying inflammatory mechanisms such as cytokine production and leukocyte migration.

Our results leave open many questions about the underlying cellular and molecular mechanisms. Traditionally the role of TRPA1 in neurogenic inflammation has been acknowledged, but the location of neuronal TRPA1, i.e. peripheral versus central, especially in pain and pain hypersensitivity remains still obscure. Further

investigations will be needed, for example by conducting experiments with multiple time points and dosing routes (peripheral, intrathecal and systemic) of TRPA1 blockers. In addition, the cell types in which TRPA1 has the greatest impact are still a matter for discussion. Indeed, TRPA1 activation has been detected in neurons and non-neuronal cells including endothelial cells, fibroblasts, mast cells and based on the present results, in chondrocytes, and in all of these cells, functional properties have been identified. TRPA1 might also be involved in intercellular signalling and this forms a challenge for experimental approaches. In animal studies such as acute paw edema, the effects obtained mainly involved the responses of whole tissues or even organisms and it is therefore impossible to distinguish between the contributions of different cell types. In contrast, conventional cell cultures include only one cell type and therefore the interaction between two cell types, such as neurons and leukocytes cannot be studied. Possible solutions could be found by utilizing co-cultures of multiple cell types or by use of cell type specific knock-out animals.

Previous studies have focused on the role of TRPA1 in pain and inhibition of TRPA1 has aimed at discovering new analgesic drugs. As discussed earlier, in the on-going clinical trials, TRPA1 inhibiting drugs have been evaluated for treating neuropathic pain, asthma and chronic obstructive pulmonary disease. Based on our findings, targeting TRPA1 could however function also as a disease modifying treatment to combat inflammation. According to our data in experimental disease models, drugs inhibiting TRPA1 activation could be beneficial in diseases such as gout flare, osteoarthritis and allergic inflammation. Various other groups have also reported positive results in treating experimental inflammatory diseases such as colitis, allergic asthma and atopic dermatitis with TRPA1 blockers (Caceres et al., 2009; Engel et al., 2011; B. Liu et al., 2013). Once actual clinical studies have been conducted, it should be possible to determine in which conditions and patient groups TRPA1 inhibiting treatment could be especially beneficial. It is possible that TRPA1 inhibiting drugs will be a symptomatic and disease-modifying option in the treatment of inflammatory diseases.

6 Summary and Conclusions

The aim of the present series of studies constituting this doctoral thesis was to reveal the role of TRPA1 activation in various experimental inflammatory conditions. A significant proinflammatory role of TRPA1 activation was found in several mouse models, i.e. experimental carrageenan-induced acute paw inflammation, MSU crystal-induced gouty arthritis and inflammation, MIA-induced model of osteoarthritis and inflammation and ovalbumin-induced acute allergic inflammation. The results showed that none of the stimulants used to induce the inflammation were direct TRPA1 channel openers, but TRPA1 was activated most likely by endogenous compounds produced at the site of inflammation. These findings have extended the role of TRPA1; it no longer is simply a nociceptor and chemosensor, instead it should be considered as a significant endogenous mechanism regulating the amplification of the inflammatory response. Finally, a novel TRPA1 blocker, pinosylvin, was observed to inhibit TRPA1-induced responses both *in vitro* and *in vivo*.

The major findings and conclusions are:

1. Carrageenan-induced acute inflammatory edema is inhibitable by blocking TRPA1 function. TRPA1-induced edema is dependent on COX function and direct TRPA1 activation *in vitro* upregulates COX-2 gene expression.

2. MSU crystals evoke gouty inflammation which is alleviated by inhibiting TRPA1 either pharmacologically or by genetic depletion. TRPA1 activation, most propably by endogenously formed factors, is involved in acute inflammatory edema, proinflammatory cytokine production, inflammatory cell accumulation and the nociception induced by MSU crystals.

3. Experimental MIA-induced osteoarthritis is alleviated in TRPA1 deficient mice as measured by joint pain and histopathological changes. The acute inflammation induced by MIA is at least partly mediated by TRPA1-induced release of neuropeptide substance P. MIA together with

IL-1β upregulates COX-2 expression in chondrocytes in a TRPA1-dependent manner, indicating that TRPA1 is expressed and functional in chondrocytes.

4. Acute allergic inflammation triggered by ovalbumin challenge either subcutaneously or topically via the eye in sensitized mice can be alleviated by treatment with a TRPA1 blocker and is impaired in TRPA1 deficient mice. Furthermore, it seems that the anti-inflammatory effect of TRPA1 blockade begins in the early-phase of the response seen as attenuated paw edema and IL-4 production. It extends also to the late-phase of the acute allergic inflammation measured as reduced eosinophil infiltration into the challenged conjunctival tissue. In addition, TRPA1 is not directly activated by ovalbumin and TRPA1 deficient mice seem to sensitize normally towards the allergen.

5. Pinosylvin inhibits TRPA1 activation as evidenced in Ca^{2+} influx and membrane current assays in HEK 293 cells transfected with TRPA1. The effect is dose-dependent and pinosylvin is efficacious when applied either prior or subsequent to the TRPA1 activator AITC. Systemic dosing of pinosylvin is able to inhibit the AITC-induced acute paw inflammation as measured by edema formation and IL-6 production.

In summary, this study has identified new possible diseases in which TRPA1 is a major factor in the pathogenesis and thus conditions in which TRPA1 blockers could be beneficial. Gout flare, inflammation associated with osteoarthritis and acute allergic inflammation could be alleviated with TRPA1 blockers. Interestingly, the therapeutic property of TRPA1 inhibition may not be limited to alleviating symptoms such as pain or edema but, since it prevents the underlying inflammatory mechanisms, it may possess a more general, disease modifying effect.

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9 Original Communications

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TRPA1 Contributes to the Acute Inflammatory Response and Mediates Carrageenan-Induced Paw Edema in the Mouse

Lauri J. Moilanen[1], Mirka Laavola[1], Meiju Kukkonen[1], Riku Korhonen[1], Tiina Leppänen[1], Edward D. Högestätt[2], Peter M. Zygmunt[2], Riina M. Nieminen[1] & Eeva Moilanen[1]

[1]The Immunopharmacology Research Group, University of Tampere School of Medicine and Tampere University Hospital, Tampere, Finland, [2]Department of Clinical Chemistry and Pharmacology, Lund University and Lund University Pain Research Centre, Lund, Sweden.

Transient receptor potential ankyrin 1 (TRPA1) is an ion channel involved in thermosensation and nociception. TRPA1 is activated by exogenous irritants and also by oxidants formed in inflammatory reactions. However, our understanding of its role in inflammation is limited. Here, we tested the hypothesis that TRPA1 is involved in acute inflammatory edema. The TRPA1 agonist allyl isothiocyanate (AITC) induced inflammatory edema when injected intraplantarly to mice, mimicking the classical response to carrageenan. Interestingly, the TRPA1 antagonist HC-030031 and the cyclo-oxygenase (COX) inhibitor ibuprofen inhibited not only AITC but also carrageenan-induced edema. TRPA1-deficient mice displayed attenuated responses to carrageenan and AITC. Furthermore, AITC enhanced COX-2 expression in HEK293 cells transfected with human TRPA1, a response that was reversed by HC-030031. This study demonstrates a hitherto unknown role of TRPA1 in carrageenan-induced inflammatory edema. The results also strongly suggest that TRPA1 contributes, in a COX-dependent manner, to the development of acute inflammation.

The identification of transient receptor potential ankyrin 1 (TRPA1) as a chemosensor of potentially harmful electrophilic and non-electrophilic chemicals[1–4] has opened up new avenues in our understanding of nociception and inflammatory pain[5]. The role of TRPA1 in noxious chemosensation has attracted considerable attention with regard to the development of TRPA1 antagonists in the treatment of pain and sensory hyperreactivity, e.g. in the therapy of the urinary bladder and airway diseases[5–7].

TRPA1 is a membrane-associated cation channel which is involved in several physiological functions such as neurotransmission, cell proliferation and gene expression via Ca^{2+} influx and elevation of the cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$)[8,9]. TRPA1 belongs to the transient receptor potential (TRP) ion channel superfamily which in mammals embraces six subfamilies and 28 distinct proteins with different functions in a variety of cells and tissues. TRPA1 was first discovered in 1999 by Jaquemar and colleagues[10] and is the only member of its subfamily (TRP ankyrin) found in humans. Structurally, TRPA1 is composed of six transmembrane spanning segments with a pore domain between 5th and 6th segments. The TRPA1 intracellular N-terminus displays 14 ankyrin repeats[11] within which lies the site of activation by the covalent modification of specific cysteines[11–13].

Many of the oxidants formed in inflammatory reactions such as nitro-oleic acid[14], 4-hydroxynonenal or hydrogen peroxide[15] are endogenous agonists of TRPA1. Furthermore, a variety of exogenous agonists, for example allyl isothiocyanate (AITC)[1], one of the pungent compounds in mustard oil, have been identified. TRPA1 antagonists have also been developed and, e.g. HC-03003[16] has become a widely used experimental tool.

TRPA1 is primarily considered to be expressed in a sub-population of sensory neurons[3,11,17], but recent findings suggest that it is also present in a number of other cells, including keratinocytes, endothelial cells, synoviocytes, odontoblasts and enterochromaffin cells[18–22]. Its physiological role remained obscure until the discovery that TRPA1 is present in mouse afferent nerves and could be activated by noxious cold, indicating a role in thermal nociception[11]. TRPA1 has been associated with other physiological functions including chemosensation, hearing

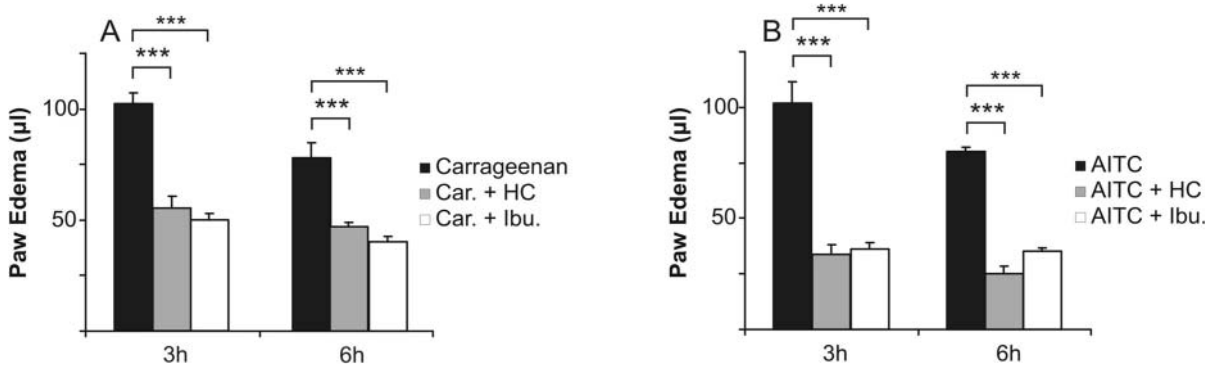

Figure 1 | TRPA1 agonist allyl isothiocyanate (AITC) and carrageenen (Car) induced an inflammatory paw edema, which could be prevented by pre-treatment with HC-030031 (HC) or ibuprofen (Ibu.). HC-030031 (300 mg/kg) or ibuprofen (100 mg/kg) was injected intraperitoneally 2 h prior to intraplantar injection of carrageenan or AITC into the hind paw. The edema was measured 3 h and 6 h after intraplantar injection and compared to the basal level. The contralateral control paw injected with saline developed no measurable edema. Mean + SEM, n=6, ***p<0.001.

and mechanical cognisance[1,5]. In addition, TRPA1 has been shown to mediate inflammatory[23] and formalin-induced pain[24], irritating effects of pungent compounds[25,26] and neurogenic inflammation[27,28]. Also, mice treated with TRPA1 antagonists and TRPA1 knock out (KO) mice were found to develop a less severe ovalbumin-induced asthma reaction than untreated wild type (WT) mice[29]. Topical treatment with mustard oil has been shown to induce local edema, an effect also blunted in TRPA1 deficient mice[25]. However, it is still far from clear if TRPA1 has a role as a modulator of the inflammatory process.

In the present study, we investigated the possible role of TRPA1 in carrageenan-induced inflammatory paw edema which is a widely used model for investigating the acute inflammatory response and novel anti-inflammatory drugs. The results show that a substantial part of the mouse paw edema triggered by carrageenan is dependent on TRPA1. Furthermore, both carrageenan and AITC-induced edemas are to a large extent inhibited by ibuprofen. These findings highlight TRPA1 as a potential drug target for novel anti-inflammatory agents that could be a valuable alternative to cyclo-oxygenase (COX) inhibitors in the treatment of certain inflammatory conditions.

Results

Intraplantar (i.pl.) injection of carrageenan induced a substantial paw edema when measured at 3 h and 6 h following injection (Fig. 1A). The contralateral paw injected i.pl. with saline exhibited no measurable edema. Likewise, the TRPA1 agonist AITC evoked a severe edema when injected in the mouse paw (Fig. 1B).

To further study the role of TRPA1 in the inflammatory edema induced by carrageenan and AITC, we treated the mice with the selective TRPA1 antagonist HC-030031[16]. The mice received 300 mg/kg of HC-030031 intraperitoneally (i.p.) 2 h before carrageenan or AITC was injected into the paw. HC-030031 treatment prevented carrageenan-induced inflammatory edema by 48% and 40% when measured at 3 h and 6 h following the carrageenan injection, respectively (Fig. 1A). As expected, HC-030031 reduced the AITC-induced edema response by 67% and 69% at 3 h and 6 h, respectively (Fig. 1B).

Next we used TRPA1-deficient mice to confirm and extend the results obtained with pharmacological blockade of TRPA1. We found that such mice developed on average 62% and 50% less edema as compared to WT mice when measured 3 h and 6 h following the i.pl. carrageenan injection (Fig. 2A). AITC induced a negligible edema in TRPA1 deficient mice (Fig. 2B) providing additional evidence that the AITC-induced response is indeed dependent on TRPA1.

The possible involvement of COX-derived prostanoids in TRPA1-mediated inflammatory edema was investigated by treating the animals with ibuprofen. Ibuprofen (100 mg/kg i.p.) given 2 h before carrageenan reduced the edema response by 51% and 49%, respectively when measured at 3 h and 6 h following carrageenan injection

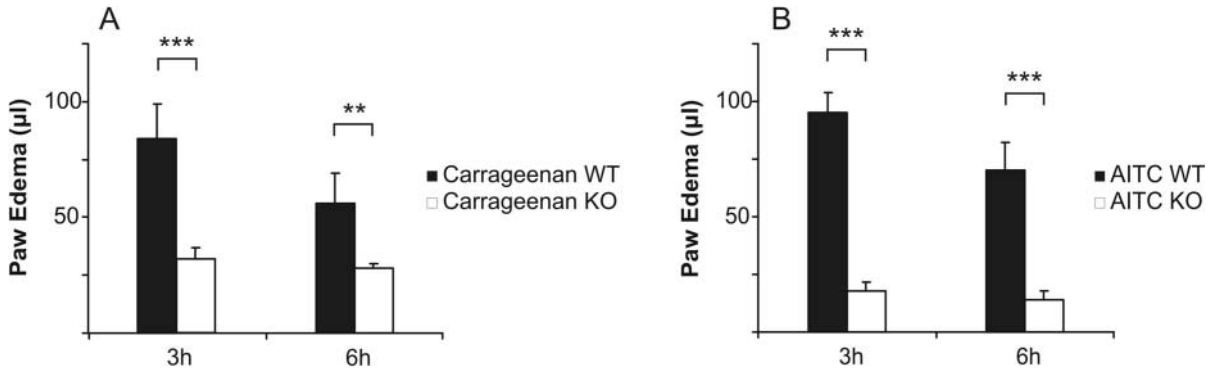

Figure 2 | In TRPA1 knock out (KO) mice, the carrageenan-induced paw edema formation was blunted when compared to the corresponding wild type (WT) mice (A). TRPA1-deficient mice showed almost no response to the TRPA1 agonist AITC (allyl isothiocyanate) in contrast to the WT mice (B). Carrageenan or AITC were injected intraplantarly. The edema was measured after 3 h and 6 h and compared to the basal level. The contralateral control paw injected with saline developed no measurable edema. Mean + SEM, n=5, **p<0.01, ***p<0.001.

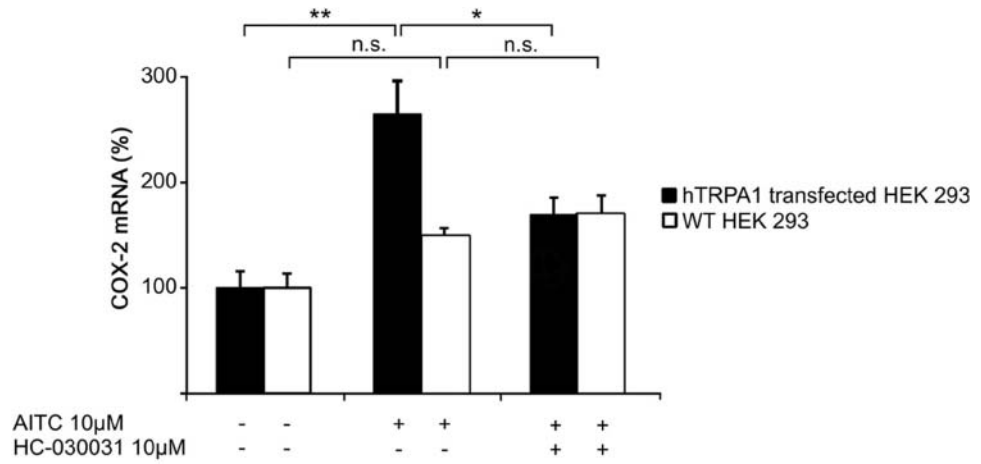

Figure 3 | HEK 293 cells transfected with human TRPA1 showed an upregulation of cyclo-oxygenase-2 (COX-2) mRNA in response to the TRPA1 agonist allyl isothiocyanate (AITC). The expression was suppressed when the TRPA1 antagonist HC-030031 (10 μM) was given before AITC. Both TRPA1 transfected and non-transfected (wild type, WT) cells were incubated with HC-030031 or vehicle for 30 min and thereafter AITC was added. After 6 h the incubations were terminated and COX-2 mRNA was assayed by real-time RT-PCR. COX-2 mRNA was normalized against GAPDH mRNA. Mean + SEM, n=4, *p<0.05, **p<0.01, n.s.=non-significant.

(Fig. 1A). Likewise, the inflammatory edema evoked by AITC was also clearly inhibited by ibuprofen pre-treatment. The mean edema was about 60% less in ibuprofen treated than in vehicle treated mice at both 3 h and 6 h time points (Fig. 1B). These results indicate that prostanoids play an important role in mediating TRPA1-induced edema.

To further investigate the link between TRPA1 and COX, we transfected HEK 293 cells with human TRPA1. The COX-2 mRNA expression was up-regulated in TRPA1 transfected HEK 293 cells but not in non-transfected cells when they were exposed to AITC. HC-030031 given 30 min before AITC reduced the extent of the up-regulation of COX-2 mRNA triggered by TRPA1 stimulation (Fig. 3), supporting the concept that COX-derived prostanoids are involved in TRPA1-induced responses.

In addition, the direct activation of TRPA1 by carrageenan was investigated in HEK 293 cells expressing human TRPA1. In those cells, exposure to carrageenan (250 μg/ml) did not evoke a change in the basal calcium level, as measured by ratiometric calcium imaging, whereas subsequent addition of AITC (100 μM) always evoked robust calcium responses (Fig. 4). Higher concentrations of carrageenan could not be tested due to viscosity problems.

Discussion

The present study confirms the effects of exogenous TRPA1 agonists on edema formation and, more interestingly, reveals a hitherto unknown role of TRPA1 in carrageenan-induced inflammatory paw edema which is a widely used model for evaluating acute inflammation and anti-inflammatory drugs. The results strongly suggest that TRPA1 has a significant role in mediating the acute inflammatory response.

Previously, TRPA1 has been shown to mediate nociceptive processes *in vivo*, such as mechanical and cold hyperalgesia[30–32], and also inflammatory pain[23,24]. A mutation in TRPA1 resulting in hyperfunction of the ion channel was recently associated with familial episodic pain syndrome[33] highlighting the significant role of TRPA1 also in human pain. Although endogenous TRPA1 agonists are produced in inflammatory reactions[14], very little is known about the possible role of TRPA1 in mediating inflammatory responses in addition to pain. Experiments conducted in knock out mice revealed that TRPA1 was involved in the pathogenesis of airway hyperreactivity and inflammation during ovalbumin-induced asthma[29]. Exposure to a TRPA1 agonist extracted from cigarette smoke was reported to cause tracheal edema which could be reversed by local administration of a TRPA1 antagonist whereas no edema formation occurred in TRPA1 KO animals[34]. In the present study, we observed that activation of TRPA1 by AITC resulted in inflammatory edema, and that a TRPA1-dependent

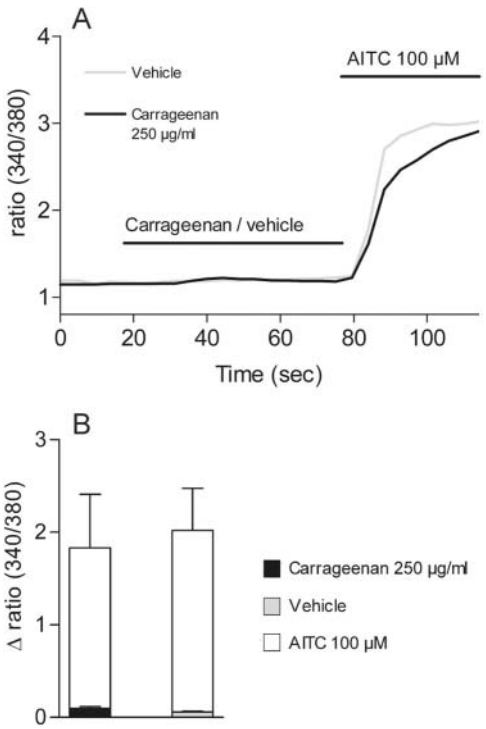

Figure 4 | In calcium imaging experiments, HEK 293 cells transfected with human TRPA1 were exposed to either carrageenan or its vehicle for 60 s and subsequently to AITC (allyl isothiocyanate) for 36 s. Whereas carrageenan or vehicle was without effect, AITC always evoked robust calcium responses as assessed by ratiometric Fura 2 imaging. Traces show the average calcium responses (A) and the bar graph (B) shows the maximum calcium responses in cells exposed to the various treatments. Mean + SEM, n=4 (each experiment was performed in duplicate or triplicate).

mechanism was involved in the formation of the classical carrageenan-induced paw edema.

The inflammatory edema following carrageenan injection involves both neurogenic and non-neurogenic mechanisms which have been strongly associated with prostaglandin production, COX-2 up-regulation and the formation of reactive nitrogen and oxygen species as well as cytokines and other inflammatory mediators[35–37]. Many such pro-inflammatory agents can sensitize or activate TRPA1 either indirectly via G protein-coupled phospholipase C and protein kinase A pathways or by directly interacting with TRPA1[5]. In the present study we showed that the TRPA1 antagonist HC-030031 clearly inhibited the development of carrageenan-induced edema while a direct TRPA1 agonist AITC evoked a carrageenan-like inflammatory response. To confirm our findings with the TRPA1 agonist and antagonist in acute inflammatory edema, we performed the experiments also in TRPA1 deficient animals. Indeed, the carrageenan-induced response was markedly attenuated in TRPA1 KO mice, and only a minor response to AITC was detected. When compared with previous reports on the acute phase of carrageenan-induced mouse paw edema, TRPA1 deficient mice showed a quantitatively similar attenuated response as mice lacking Akt1[38], endothelial nitric oxide-synthase[39] and the tumor necrosis factor α receptor 1[40,41]. Taken together, our results support the conclusion that TRPA1 activation is involved in the pathogenesis of acute inflammatory edema in response to carrageenan.

Both mouse and human TRPA1 is inhibited by HC-030031, which is considered to be a selective TRPA1 antagonist as it is inactive on 48 other essential proteins involved in pain and inflammation including COX-2 and TRPV1[5,16,24]. AITC is present in many naturally occurring plant-derived sources and has been shown to have antimicrobial[42,43], cytostatic and cancer protective[44] properties linked to multitudinous cellular targets. Whether TRPA1 mediates these effects is, however, unclear. As shown in the present study, AITC evoked edema in wild type but not in TRPA1 deficient animals, and in WT mice the AITC-response was inhibited by the TRPA1 antagonist HC-030031. These data together suggest that the AITC-induced edema response found in the present study was indeed mediated by TRPA1.

Given that TRPA1 is a highly promiscuous chemosensor[5,45], one cannot rule out the possibility that TRPA1 may also recognize carrageenan. Due to its high viscosity, we could not test the direct effect of carrageenan in concentrations above 250 μg/ml on TRPA1. This concentration is 40-60 times lower than stock solutions normally used for intraplantar injections[37] and 8 times lower than the concentration known to induce inflammation[46]. However, the injected carrageenan is quickly diluted within the tissue and hence the local concentrations may well correspond to the *in vitro* test concentration that we found inactive at TRPA1 expressed in HEK 293 cells. Thus, we believe that the inflammation triggered by carrageenan is most likely not initiated by a direct interaction with TRPA1, but rather involves TRPA1 at some subsequent step in the inflammatory cascade.

The ability of ibuprofen to reduce both carrageenan and AITC-induced edema suggests that it inhibits COX downstream of TRPA1. Cellular calcium influx through TRPA1 would trigger the calcium-dependent release of prostaglandins and other pro-inflammatory agents that may further sensitize TRPA1 and increase its cell surface expression as occurs in sensory neurons[47,48]. It is also possible that TRPA1 activation increases prostaglandin production through enhanced COX-2 expression. This is supported by our *in vitro* finding that activation of TRPA1 by AITC can up-regulate the COX-2 transcript in HEK 293 cells. Accordingly, a significant role of $[Ca^{2+}]_i$ in regulating COX-2 expression in macrophages was recently reported[49]. Together these events would generate and maintain a TRPA1 and COX-dependent inflammation. Our finding that the TRPA1 antagonist HC-030031 is as effective as the COX inhibitor ibuprofen to inhibit the development of carrageenan and AITC-induced edema is promising, and may help to develop safer anti-inflammatory drugs than existing COX inhibitors.

An interesting question remains on the mechanisms behind TRPA1-mediated inflammatory edema which may involve neurogenic and/or non-neurogenic components. TRPA1 activation in afferent nerve endings may lead to a focal release of bioactive compounds such as calcitonin gene-related peptide (CGRP) and substance P which play a major role in neurogenic inflammation and exert vascular effects which may contribute to the formation of inflammatory edema[5]. However, spinal TRPA1 may also regulate the peripheral responses through retrograde afferent signaling[30]. In addition, activation of TRPA1 on vascular sensory neurons and/or endothelium[50] can lead to vasodilatation and increased vascular permeability and edema formation[51]. Since endothelium is also a known source of vasodilating and edema-evoking prostaglandins and other prostanoids, this could provide an explanation for the link between TRPA1 and COX, as observed in the present study.

In humans, TRPA1 is present on nociceptive nerve endings and in non-neuronal cells such as keratinocytes and fibroblasts, from which prostaglandins and other mediators may be released after TRPA1 activation[52,53]. Therefore, the mouse carrageenan-induced paw edema may be a useful model to identify novel compounds and drugs targeting TRPA1 in humans.

Our study demonstrates a significant role for TRPA1 in the development of acute inflammation, and suggests that TRPA1 antagonists may have anti-inflammatory properties in addition to their previously recognized analgesic effects in inflammatory pain.

Methods

Wild type and TRPA1-deficient C57BL/6 mice were used in the experiments. TRPA1-deficient mice and the corresponding WT mice were originally obtained from Dr David Julius (UCSF) and back-crossed in the laboratory of EDH and PMZ (Lund University, Sweden). TRPA1 genotype was confirmed by PCR. The mice were housed under standard conditions (12:12 h light-dark cycle, 22±1°C, 50–60% humidity) with food and water provided *ad libitum*.

The mice were divided into groups of six and were treated with 150 μl of saline (vehicle), ibuprofen (100 mg/kg, Sigma Chemical Co., St. Louis, MO, USA) or HC-030031 (300 mg/kg, Sigma Chemical Co.) i.p. 2 h before administering carrageenan or AITC. Before inducing the paw edema, the mice were anesthesized by i.p. injection of 0.5 mg/kg medetomidine (Domitor®, Orion Oyj, Espoo, Finland) and 75 mg/kg ketamine (Ketalar®, Pfizer Oy Animal Health, Helsinki, Finland). To induce the edema, mice received ipsilaterally i.pl. injection (30 μl) of either λ-carrageenan (15 mg/ml, Sigma Chemical Co.) or AITC (6.66 mM, Sigma Chemical Co.) into the hind paw. The control paw was injected with 30 μl of saline and developed no measurable edema. The study was approved by the National Animal Experiment Board.

Edema was measured before and at 3 h and 6 h after carrageenan or AITC injection by using a plethysmometer (Ugo Basile, Comerio, Italy). Edema is expressed as the difference between the carrageenan or AITC treated paw at the time indicated and the basal level.

HEK 293 human embryonic kidney cells (American Type Culture Collection, Manassas, VA, USA) were cultured in Eagle's Minimum Essential Medium (EMEM) supplemented with 10% heat-inactivated fetal bovine serum, sodium bicarbonate (1.5%), sodium pyruvate (1 mM), non-essential amino acids (1 mM each) (all from Lonza , Verviers SPRL, Verviers, Belgium), penicillin (100 U/ml), streptomycin (100 μg/ml) and amphotericin B (the last three compounds from Invitrogen, Paisley, UK) at 37°C in 5% CO_2. The cells were transfected using 0.42 μg/cm² human TRPA1 plasmid DNA (pCMV6-XL4 by Origene Rockville, MD, USA) with Lipofectamine 2000 (Invitrogen) according to the manufacturer's directions. After 24 h of transfection, HC-030031 (10 μM) or solvent (control) was added to the cells in fresh culture medium 30 min prior to the activation of TRPA1 by AITC (10 μM). The cells were then incubated for 6 h before being harvested for RNA extraction. Similar experiments were carried out by using non-transfected (wild type, WT) HEK 293 cells.

Total RNA extraction was carried out with the use of GenElute™ Mammalian Total RNA Miniprep Kit (Sigma). Reverse-transcription of RNA to cDNA and quantitative RT-PCR reactions were performed as previously described[54]. TRPA1 expression was measured by using TaqMan Gene Expression Assay (Applied Biosystems, Foster City, CA, USA). COX-2 and GAPDH primers and probes were identical to those previously described[55]. COX-2 mRNA levels were normalized against GAPDH.

Fluorometric calcium imaging was used to study the effect of carrageenan on human TRPA1 expressed in HEK 293 cells. The cells were plated in 96-well black-walled plates (Costar, Cambridge, MA, USA) and loaded with Fura 2-AM (1 μM,

Invitrogen), probenecid (2 mM, Sigma Chemical Co.) and pluronic acid (20%, Invitrogen) for 1 h at 37°C. The cells were then washed with physiological buffer solution (PBS), containing 140 mM NaCl, 5 mM KCl, 10 mM glucose, 10 mM HEPES, 2 mM $CaCl_2$ and 1 mM $MgCl_2$, and allowed to equilibrate for a period of 30 min in the dark before the start of the experiments. The intracellular calcium concentration was determined at 25°C in a Flexstation 3 (Molecular Devices, Sunnyvale, CA, USA). Basal emission (510 nm) ratios with excitation wavelengths of 340 nm and 380 nm were measured and changes in dye emission ratio (Δ ratio) determined at various times after compound addition.

Results are expressed as mean ± standard error of mean (SEM). Statistical analysis was carried out by using Student's *t*-test or one-way ANOVA with Bonferroni's multiple comparisons test and results were considered significant at *$p<0.05$, **$p<0.01$ and ***$p<0.001$.

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Author contribution

LJM participated in the animal and laboratory experiments, calculated the results and statistics and drafted the manuscript. ML, MK, RK, TL and RMN participated in the animal and the laboratory experiments. EDH and PMZ provided the TRPA1 knock out and corresponding wild type animals and carried out the calcium imaging experiments. EM conceived and supervised the study and helped to draft the manuscript. All authors were involved in the planning of the study and in writing the manuscript.

Additional information

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RESEARCH ARTICLE

Urate Crystal Induced Inflammation and Joint Pain Are Reduced in Transient Receptor Potential Ankyrin 1 Deficient Mice – Potential Role for Transient Receptor Potential Ankyrin 1 in Gout

Lauri J. Moilanen, Mari Hämäläinen, Lauri Lehtimäki, Riina M. Nieminen, Eeva Moilanen*

The Immunopharmacology Research Group, University of Tampere School of Medicine and Tampere University Hospital, Tampere, Finland

* eeva.moilanen@uta.fi

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Abstract

Introduction

In gout, monosodium urate (MSU) crystals deposit intra-articularly and cause painful arthritis. In the present study we tested the hypothesis that Transient Receptor Poten-tial Ankyrin 1 (TRPA1), an ion channel mediating nociceptive signals and neurogenic in-flammation, is involved in MSU crystal-induced responses in gout by utilizing three experi-mental murine models.

Methods

The effects of selective pharmacological inhibition (by HC-030031) and genetic depletion of TRPA1 were studied in MSU crystal-induced inflammation and pain by using 1) spontaneous weight-bearing test to assess MSU crystal-induced joint pain, 2) subcutaneous air-pouch model resembling joint inflammation to measure MSU crystal-induced cytokine production and inflammatory cell accumulation, and 3) MSU crystal-induced paw edema to assess acute vascular inflammatory responses and swelling.

Results

Intra-articularly injected MSU crystals provoked spontaneous weight shift off from the affected limb in wild type but not in TRPA1 knock-out mice referring alleviated joint pain in TRPA1 deficient animals. MSU crystal-induced inflammatory cell infiltration and accumulation of cytokines MCP-1, IL-6, IL-1beta, MPO, MIP-1alpha and MIP-2 into subcu-taneous air-pouch (resembling joint cavity) was attenuated in TRPA1 deficient mice and in mice treated with the selective TRPA1 inhibitor HC-030031 as compared to control animals. Further, HC-030031 treated and TRPA1 deficient mice developed tempered inflammatory edema when MSU crystals were injected into the paw.

Competing Interests: The authors have declared that no competing interests exist.

Conclusions

TRPA1 mediates MSU crystal-induced inflammation and pain in experimental models supporting the role of TRPA1 as a potential mediator and a drug target in gout flare.

Introduction

Gout is an increasing inflammatory disease with a prevalence of 1–2%. Gout flares are characterized by acute burning arthritis with local hyperalgesia and pain caused by monosodium urate (MSU) crystals accumulated into the affected joint. [1] In inflammatory cells, non-soluble MSU crystals trigger the formation of reactive oxygen species (ROS) and activate inflammatory signaling cascades such as phosphoinositide 3-kinase (PI3K) and nuclear factor- κB (NF-κB) pathways, and NALP3 inflammasome. Activation of NALP3 results in caspase-1 mediated cleavage of pro-interleukin-1β to the functional inflammatory cytokine interleukin-1β (IL-1β). [2,3] IL-1β has been investigated as a potential therapeutic target in acute gouty arthritis and it has been found to mediate partly, but not fully, inflammatory and analgesic responses in MSU crystal-induced inflammation [4].

Transient Receptor Potential Ankyrin 1 (TRPA1) is a Ca^{2+} permeable ion channel involved in cold allodynia, nociception, and according to the recent findings, also in inflammation [5–10]. Since its discovery in 1999 [11] TRPA1 has drawn increasing interest as a therapeutic target to treat neuropathic and inflammatory pain [12,13]. TRPA1 was originally discovered in sensory neurons but later the expression and function of TRPA1 in various non-neuronal cells has been established [5]. TRPA1 is activated by noxious cold and a spectrum of naturally occurring irritating compounds, e.g. allyl isothiocyanate in mustard oil or allicin in garlic. Interestingly, many reactive molecules formed in inflammation, such as hydrogen peroxide, 4-hydroxynonenal, nitrooleic acid and some arachidonic acid metabolites also activate TRPA1 [5,14,15]. Furthermore, inflammatory signaling pathways, such as protein kinase A and phospholipase C, are known to sensitize TRPA1 [16]. In addition to the regulation of analgesic signals, activation of neuronal TRPA1 contributes to neurogenic inflammation by releasing proinflammatory neuropeptides calcitonin gene related peptide and substance P [5]. Together with the published data [17,18] our recent findings in a widely used animal model in anti-inflammatory drug research, i.e. carrageenan-induced paw inflammation [19], further suggest that TRPA1 is not only a target of exogenous noxious signals but also a significant endogenous mechanism involved in amplification of acute inflammation [19].

Pharmacological blockade and genetic depletion of TRPA1 have shown beneficial effects in several models of hyperalgesia and pain as well as in acute inflammation [5,6,8]. Therefore we hypothesized that TRPA1 may contribute to MSU crystal-induced inflammation and pain in gout flare. The aim of the present study was to address the hypothesis by investigating the effects of pharmacological inhibition and genetic depletion of TRPA1 in animal models evaluating MSU crystal-induced proinflammatory cytokine production, inflammatory edema and joint pain.

Methods

Animals

Wild type (WT) and TRPA1 knock-out (KO) B6;129P-Trpa1(tm1Kykw)/J mice (Charles River Laboratories, Sulzfeld, Germany) were used in the experiments. Mice were housed under

standard conditions (12–12h 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 (approval number ESAVI/5250/04.10.03/2012, granted on September 3, 2012).

Intraperitoneal injection 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 for anesthesia. Animals were sacrificed after experiments by carbon monoxide followed by cranial dislocation. Reagents were purchased from Sigma Chemical Co., St. Louis, MO, USA unless otherwise indicated.

Studied Animal Groups

To study drug effects, WT mice were dosed orally with the selective TRPA1 antagonist HC-030031 [20] (300 mg/kg), with the control compound dexamethasone (2 mg/kg) with known anti-inflammatory activity or with the vehicle 2h prior to the experiments. All drugs used were diluted in 75% polyethylene glycol and given by gastric gavage in a volume of 250 μl. The dose of HC-030031 was based on our previous studies and literature [19,21,22]. Effects of genetic depletion of TRPA1 were studied by comparing responses in TRPA1 KO and corresponding WT mice.

Subcutaneous Air-Pouch Test

Subcutaneous air-pouch was created by injecting 3 ml (1st day) and 1.5 ml (3rd day) of sterile air into the dorsal skin of the studied mice under anaesthesia and after 7 days a synovial-like epithelium was present in the air-pouch [23]. The inflammation was induced by injecting 3 mg of MSU crystals prepared as described below diluted in 1 ml of sterile endotoxin free phosphate-buffered saline (PBS) into the air-pouch of the anesthetized mice. After 6h the mice were sacrificed and the exudate was harvested for cell-counting by hemocytometer and for cytokine measurements. Monocyte chemotactic protein-1 (MCP-1), interleukin-6 (IL-6), interleukin-1β (IL-1β), myeloperoxidase (MPO), macrophage inflammatory protein-1α (MIP-1α) and macrophage inflammatory protein-2 (MIP-2) were measured by enzyme-linked immunosorbent assay (ELISA) (R&D Systems Europe Ltd, Abindgon, UK).

Paw Edema Test

Inflammatory paw edema was induced by injecting 0.5 mg of MSU crystals diluted in 40 μl of sterile endotoxin free PBS into the hind paw of anesthetized mice. Contralateral paw was injected with the corresponding volume of the vehicle and developed no measurable edema. The paw volume was measured up to 6h with plethysmometer (Ugo Basile, Comerio, Italy) and compared to the baseline value.

Weight-Bearing Test

The MSU crystal-induced weight-bearing test originally described in 1986 [24,25] was triggered by injecting 0.5 mg of MSU crystals diluted in 40 μl of sterile endotoxin free PBS into the hind knee joint of anesthetized mice. Contralateral knee joint was injected with the corresponding volume of the vehicle. The willingness to bear weight on the affected joint was measured with an incapacitance meter (IITC Life Science, Woodland Hills, CA, USA) for four subsequent 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. To obtain reliable data on the weight distribution, each mouse was measured 8 times for 1 second at each time point.

Preparation of MSU Crystals

MSU crystals were prepared as previously described [26] by diluting 1.0 g of uric acid into 200 ml of aqua adjusted to pH 14.0 with NaOH by heating and blending. Next, pH was lowered to 7.0 by adding HCl and MSU was crystallized overnight at room temperature in constant shaking. The formed crystals were filtered, washed, dried and re-suspended in PBS at a concentration of 50 mg/ml. In microscopic examination the MSU crystals were 5–20 μm in length. All used equipment and liquids were endotoxin free.

Ca^{2+}-Influx Measurement

TRPA1 mediated Ca^{2+}-influx was measured in HEK293 cells [27] transiently transfected with plasmid encoding the human TRPA1 (pCMV6-XL4 from Origene, Rockville, MD, USA) as described previously [19]. Cultured cells were loaded with 4 μM fluo-3-acetoxymethyl ester and 0.08% Pluronic F-127 in Hanks' balanced salt solution (HBSS) containing 1 mg/ml of bovine serum albumin, 2.5 mM probenecid and 25 mM HEPES (pH 7.2) for 30 minutes at room temperature. The intracellular free Ca^{2+} levels were assessed by Victor3 1420 multilabel counter (Perkin Elmer, Waltham, MA, USA) at excitation/emission wavelengths of 485/535 nm [28]. In the experiments, the cells were first pre-incubated with 100 μM of the TRPA1 antagonist HC-030031 [20] or the vehicle for 30 min at +37 C°. Thereafter, 1 mg/ml of MSU crystals or 50 μM AITC was added and the measurements were continued for 30s after which a robust Ca^{2+}-influx was induced by application of 1 μM of control ionophore compound ionomycin. The concentrations of AITC and HC-030031 were based on our dose-response studies and literature [29,30].

Statistical Analysis

Results are expressed as mean ± standard error of the mean (SEM). Data were analyzed with SPSS version 17.0 for Windows software (SPSS Inc, Chicago, IL, USA) by using Student's *t*-test or one-way ANOVA with Bonferroni's or Dunnett's multiple comparison test.

Results

Monosodium urate (MSU) crystals induced an acute inflammatory response when injected into the subcutaneous air-pouch of studied mice. The response was characterized by accumulation of cells and inflammatory cytokines monocyte chemotactic protein-1 (MCP-1), interleukin-6 (IL-6), interleukin-1β (IL-1β), myeloperoxidase (MPO), macrophage inflammatory protein-1α (MIP-1α) and macrophage inflammatory protein-2 (MIP-2) (S1 Table). The acute inflammatory response induced by injection of MSU crystals into subcutaneous air-pouch was remarkably inhibited by treatment with the selective TRPA1 antagonist HC-030031 or with dexamethasone which was used as a control compound with known anti-inflammatory activity. Interestingly, HC-030031 treatment significantly inhibited the total accumulation of cells (42% inhibition, $p<0.05$) and proinflammatory cytokines MCP-1, MPO, MIP-1α, MIP-2 (37–60% inhibition, $p<0.05$ or $p<0.01$) and a similar trend was seen in IL-6 (42% inhibition, $p = 0.129$) and IL-1β (33% inhibiton, $p = 0.067$). As expected, the total accumulation of cells and proinflammatory mediators was significantly attenuated also by dexamethasone. Comparable results to those seen in pharmacological inhibition of TRPA1 were also observed when responses in TRPA1 KO and WT mice were compared. In KO mice the total amounts of IL-6,

IL-1β, MPO, MIP-1α, and MIP-2 were attenuated in a statistically significant manner (45–83% reduction, $p<0.05$ or $p<0.01$) as compared to the corresponding WT mice and a non-significant reduction in MCP-1 production (33% reduction, $p = 0.148$) was observed. (Fig. 1A and 1B)

Intraplantar injection of MSU crystals induced an acute inflammatory edema (Fig. 2). The MSU crystal-induced paw edema was clearly attenuated by the treatment with the selective TRPA1 antagonist HC-030031 (61% inhibition at 2h, $p<0.05$) or the positive control compound dexamethasone. Accordingly, TRPA1 KO mice developed significantly less severe edema than corresponding WT mice (57% reduction at 2h, $p<0.05$). (Fig. 2A and 2B)

In the weight-bearing test indicating joint pain, WT mice developed a clear reduction in spontaneous weight-bearing on the affected limb when measured 1, 2 and 3 days after an intra-articular injection of MSU crystals and the weight distribution was normalized at the 4^{th} day. Interestingly, injection of MSU crystals did not induce any weight distribution change in TRPA1 deficient mice. (Fig. 3)

Direct activation of TRPA1 by MSU crystals was excluded by studying Ca^{2+}-influx in TRPA1-transfected HEK293 cells. MSU crystals did not evoke Ca^{2+}-influx to the TRPA1-transfected cells indicating that MSU crystals do not function as a direct TRPA1 agonist. In contrast, introduction of AITC to the cells induced a strict increase in the intracellular Ca^{2+} concentration which was inhibited by the TRPA1 antagonist HC-030031. (Fig. 4A-C)

Discussion

The present results show that TRPA1 has a significant role in the development of MSU crystal-induced joint pain, inflammatory mediator production and edema formation as evidenced by the effects of pharmacological blockade and/or genetic depletion of TRPA1 in murine models.

In the present study, three models of MSU crystal-induced response were investigated. The air-pouch inflammation model is a widely used model to mimic synovial inflammation enabling the harvest of inflammatory exudate which is extremely difficult to sample reliably from the very small mouse joints. The results show that TRPA1 mediates the MSU crystal-induced accumulation of inflammatory cells (mainly neutrophils), and inflammatory cytokines into the air-pouch. Secondly, we used paw inflammation model to measure inflammatory edema related to soft-tissue inflammation, and found that TRPA1 mediates also the MSU crystal-induced inflammatory edema formation which is linked to vascular leakage. Thirdly, we used weight-bearing test to estimate joint pain as nociceptive responses were not possible measurements in the two previous models as their license allows to use only anesthetized mice in those experiments. According to the results of the weight-bearing test, TRPA1 mediates also the MSU crystal-induced joint pain. Intriguingly, Ferreira and Geppetti with their co-workers published very recently two articles reporting that activation of TRPA1 contributes to MSU crystal-induced responses when MSU crystals were injected in the rodent paw [17] or ankle joint [18]. They found that TRPA1 antagonist was able to inhibit MSU crystal-induced acute edema, nociception, production of IL-1β and myeloperoxidase and accumulation of neutrophils. They also discovered that genetic depletion of TRPA1 reduced the acute nociceptive and edematogenic effects of TRPA1. Those findings highly support the results of the current study. Further, the present results extend the previous knowledge by providing information on air-pouch model and on a broad range of proinflammatory cytokines, and by extending the studied time span of pain-like behavior resulting from MSU crystal injection as demonstrated in the spontaneous weight bearing test. These three studies beautifully complement and strengthen each other and highlight the previously unknown role of TRPA1 in the development of MSU crystal-induced inflammation and pain applicable for the pathogenesis and drug development for gout flare.

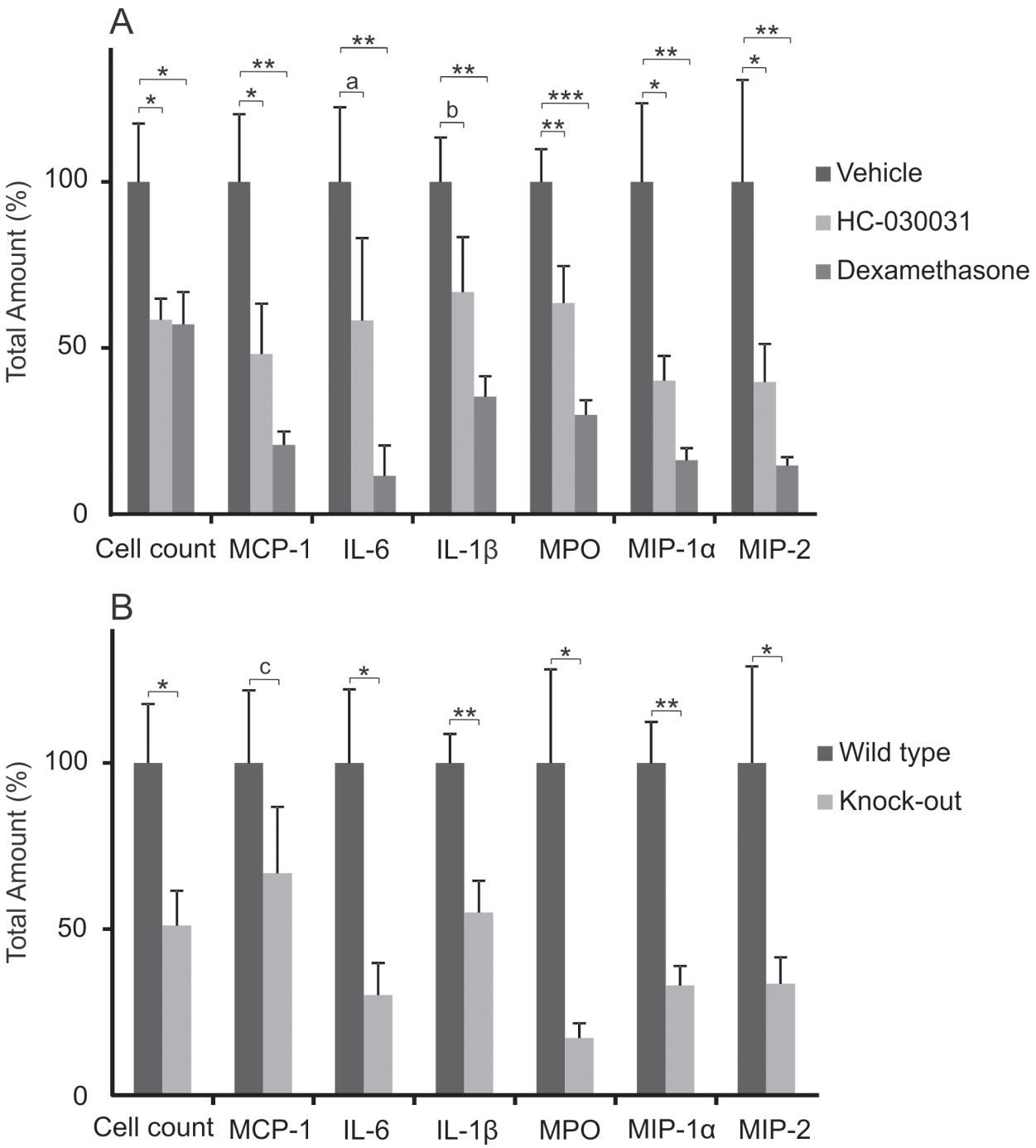

Fig 1. Effect of TRPA1 Activation in MSU Crystal-Induced Air-Pouch Test. Treatment with TRPA1 antagonist HC-030031 (300 mg/kg) or dexamethasone (2 mg/kg) inhibited monosodium urate (MSU) crystal-induced production of proinflammatory cytokines MCP-1, IL-6, IL-1β, MPO, MIP-1α and MIP-2 and accumulation of cells in the synovial joint resembling subcutaneous air-pouch model in the mouse (A). Accordingly, genetic depletion of TRPA1 led to an attenuated inflammatory response when compared to corresponding wild type mice (B). The studied drugs were given orally 2h prior to 3 mg of MSU crystals diluted in 1 ml of endotoxin free phosphate buffered saline were injected into the air-pouch. The exudate was harvested 6h after the MSU crystal injection and cells were counted using hemocytometer and the cytokines were analysed using ELISA. Results are displayed as total amount of cells or cytokines per air-pouch. The mean amount of the vehicle treated mice was set as 100% and the other values are related to that. The results are expressed as mean + SEM, n = 7–8, a indicates $p = 0.129$, b indicates $p = 0.067$, c indicates $p = 0.148$, * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$.

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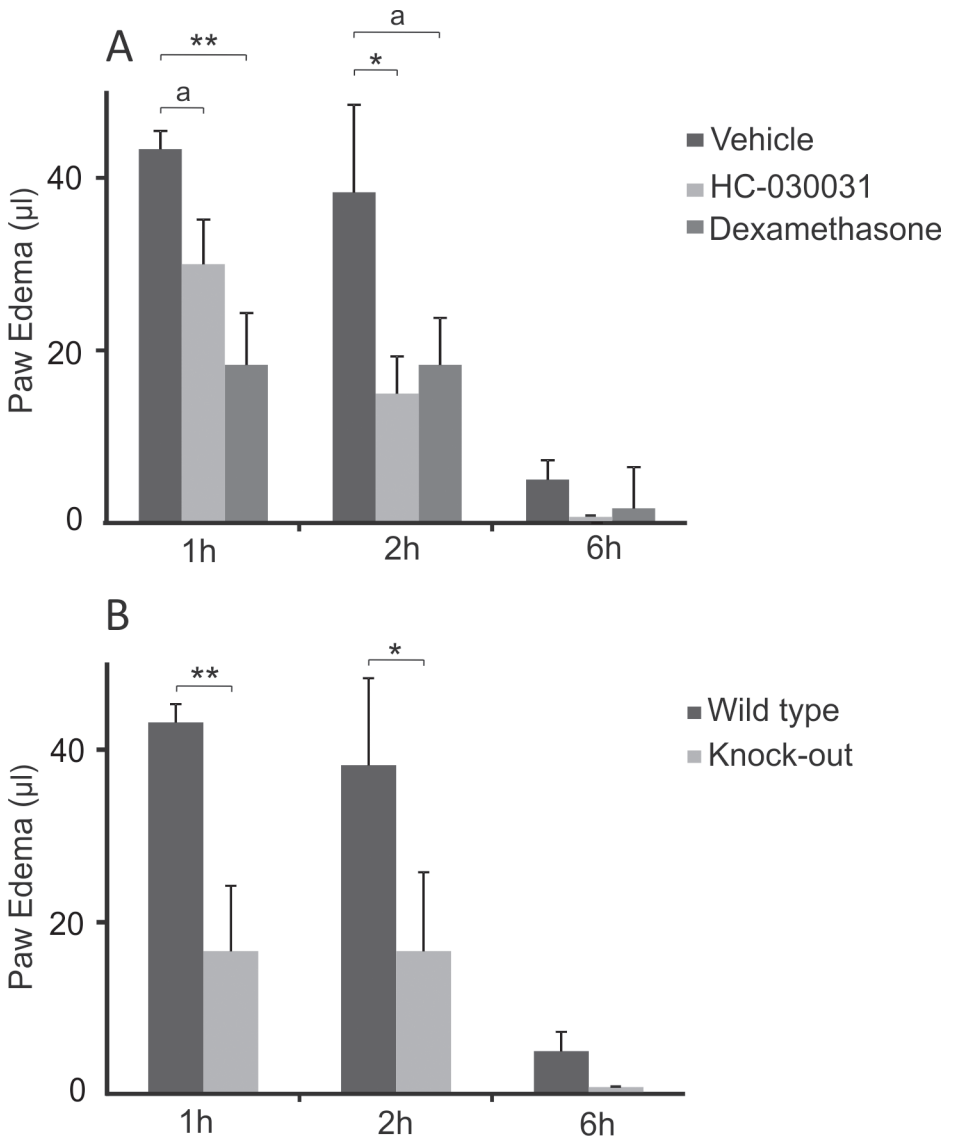

Fig 2. Effect of TRPA1 Activation in MSU Crystal-Induced Inflammatory Paw Edema. Treatment with TRPA1 antagonist HC-030031 (300 mg/kg) or dexamethasone (2 mg/kg) inhibited mouse paw edema formation induced by an injection of monosodium urate (MSU) crystals (A). In non-treated mice, TRPA1 deficiency caused an alleviated edema formation compared to the corresponding wild type mice (B). The studied drugs were given orally 2h prior to the initiation of the experiment by injecting 0.5 mg of MSU crystals in 40 µl of endotoxin free phosphate buffered saline into the mouse hind paw. The paw volume was measured with a plethysmometer before and up to 6h after MSU crystal injection. The contralateral control paw injected with the vehicle 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 + SEM, n = 5–6, a indicates $p = 0.057$, * = $p<0.05$, ** = $p<0.01$.

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TRPA1 activation has proved to mediate nociception and neurogenic and inflammatory pain in various experimental models [5,6,13]. Also, TRPA1 hyperfunction due to genetic mutation was recently reported to be associated with a severe familiar episodic pain syndrome proving the significant role of TRPA1 in human pain [31]. The present results together with those reported by Ferreira's and Geppetti's groups [17,18] show that TRPA1 mediates joint pain in a mouse model of MSU crystal-induced arthritis adding MSU crystals into the list of painful

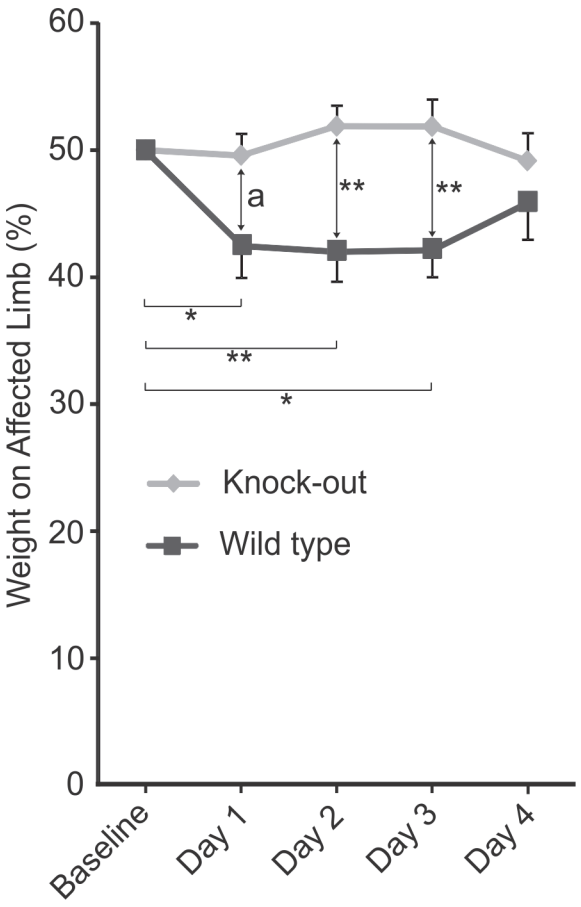

Fig 3. Effect of TRPA1 Activation in MSU Crystal-Induced Spontaneous Weight-Bearing Test. Spontaneous weight-bearing on hind limb knee joint injected with monosodium urate (MSU) crystals indicative of joint pain was altered in wild type but not in TRPA1 deficient mice. The difference between the groups was also significant. An injection of 0.5mg of MSU crystals diluted in 40 μl of endotoxin free phosphate buffered saline into the knee joint was performed and the mice were measured for spontaneous distribution of weight between hind limbs with an incapacitance meter for four subsequent days and referred to the basal level. The contralateral knee was injected with the vehicle only and the measurer was blinded for the affected limb. Results are displayed as the percentage of weight bore by the affected limb and given as mean + SEM, n = 7–10, * = $p<0.05$, ** = $p<0.01$.

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compounds mediating their effects through indirect activation of TRPA1 channels. Further, the results strongly suggest TRPA1 as a mediator and drug target to treat painful gout flares in man.

Several irritating exogenous compounds known to cause inflammatory edema in the skin, gut or respiratory track have appeared as direct activators of TRPA1 [5,6]. Interestingly, in addition to sensing exogenous irritating/proinflammatory compounds, TRPA1 has also been reported to be an endogenous mechanism mediating inflammatory edema highlighted by the fact that many compounds not directly interacting with TRPA1 cause an inflammatory reaction yet dependent on the activation of TRPA1 [5,6,19]. The TRPA1 triggered inflammatory edema – which is usually associated with hyperalgesia – has been proposed to be connected to several classical inflammatory mechanisms such as mast cell degranulation, neutrophil migration, release of histamine, serotonin and adrenalin and production of prostaglandins [19,32]. Furthermore, activation of TRPA1 has been reported to enhance the expression of inflammatory genes including IL-1β and the prostaglandin producing enzyme cyclooxygenase-2 in certain

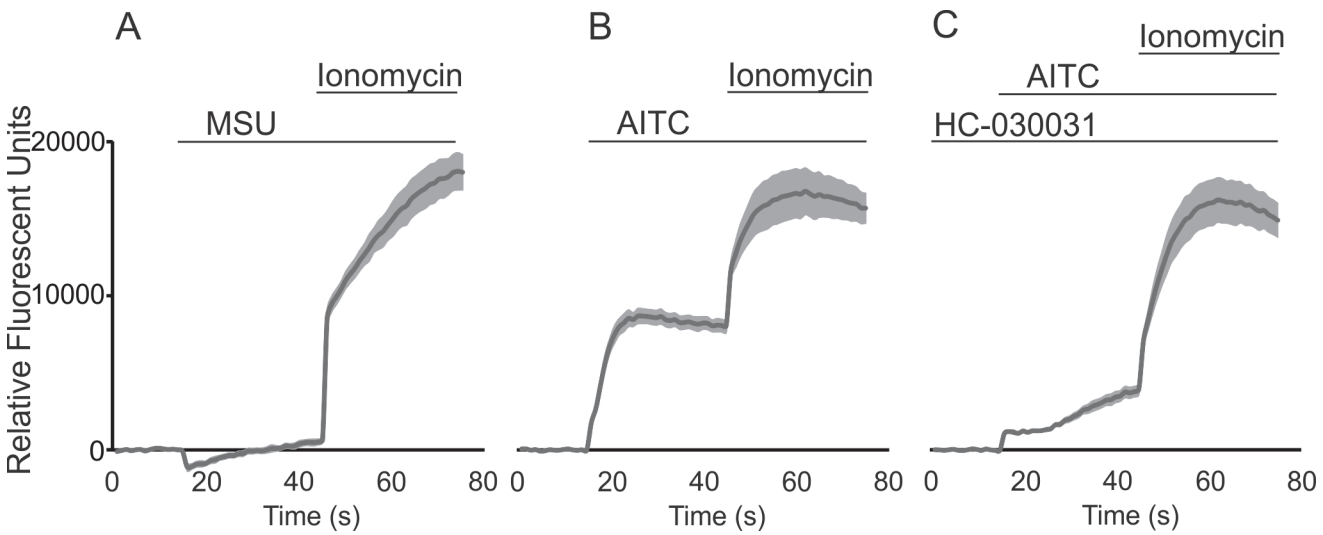

Fig 4. Effect of MSU Crystals in TRPA1-Mediated Ca^{2+}-Influx in HEK293 Cells. Monosodium urate (MSU) crystals did not evoke TRPA1-mediated Ca^{2+}-influx (A) whereas the known TRPA1 agonist allyl isothiocyanate (AITC) induced a robust Ca^{2+}-influx (B) which was inhibited by pre-treatment with the selective TRPA1 antagonist HC-030031 (C). HEK293 cells were transfected with plasmids encoding TRPA1 and loaded with Fluo-3 AM as described in the Methods. The intracellular Ca^{2+} concentration was assessed by Victor3 multilabel counter at excitation/emission wavelengths of 485/535 nm at 1/s frequency. The cells were first pre-incubated with 100 μM HC-030031 (C) or the vehicle (A-B) for 30 min at +37 C°. In the experiments (A-C) basal fluorescence was first measured for 15s and thereafter 1 mg/ml of MSU crystals (A) or 50 μM AITC (B-C) was added and the measurement was continued for 30s after which 1 μM of the control ionophore compound ionomycin was introduced to the cells. The seen transient minor fall in the relative fluorescent units after the application of MSU (A) is regarded as an artifact as a similar phenomenon is often seen following an application of inert compounds or solvents into the wells. The results are normalized against background and expressed as mean (drak gray line) ± SEM (light gray shadowing), n = 6.

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experimental models [19,33,34]. Accordingly, we found in the present study that pharmacological inhibition and genetic depletion of TRPA1 reduced the production of several MSU crystal-induced chemokines and inflammatory cytokines as measured in the air-pouch model. Even though mechanisms related to TRPA1-triggered changes in gene expression are not fully understood, TRPA1-induced increase in intracellular Ca^{2+} concentration may directly mediate the effect as changes in intracellular Ca^{2+} levels are known to regulate the transcription of some inflammatory genes [35–37]. Against this background, our present findings, in which inhibition or depletion of TRPA1 significantly reduced edema formation and accumulation of proinflammatory cytokines and inflammatory cells in response to MSU crystals in synovial joint resembling subcutaneous air-pouch and in subcutaneous soft-tissue are reasonable and together with the results described recently [17,18] support the role of TRPA1 as a mediator of inflammatory responses in acute gouty arthritis.

Presentation of MSU crystals to the inflammatory cells triggers several inflammatory mechanisms including ROS production and activation of PI3K and NF-κB pathways and NALP3 inflammasome. Activation of NALP3 leads to the release of powerful proinflammatory cytokine IL-1β [2,3]. In animal models, elimination of IL-1β has been reported to cause a clear but not a total inhibition of inflammation and pain suggesting the presence of additional mechanisms mediating the responses [4]. The present study proposes TRPA1 as such a mechanism. However, in the intracellular Ca^{2+} measurements MSU crystals were found not to directly activate TRPA1. An interesting hypothetical link between TRPA1 and MSU crystals lies, however, in the fact that MSU crystals induce oxidative stress and ROS production [2,3]. Reactive oxygen species are involved in the activation of NALP3 inflammasome and other cellular responses to MSU crystals [2,3]. Notably, multiple products of oxidative stress e.g. hydrogen peroxide and 4-hydroxynonenal are also known to activate TRPA1 which could amplify the inflammatory

response either by NALP3 dependent or independent mechanism following exposure to MSU crystals [6,15,17,18].

TRPA1 mediated inflammation is frequently explained by the neuronal expression of TRPA1 and the release of proinflammatory neuropeptides following its activation [6,13]. Calcitonin gene related peptide and substance P are best known of those neuropeptides which mediate the neurogenic inflammation triggered by activation of neuronal TRPA1. Curiously, the release of substance P from the surrounding neurons has been previously proposed in an experimental MSU crystal-induced arthritis [38] promoting neurogenic inflammation also as a potential mechanism involved in the formation of gouty arthritis. The role of neuronal mechanism in the development or MSU crystal-induced inflammation is supported by the finding that neonatal dysfunctionalization of peptidergic nerve fibers by capsaicin attenuates the pain induced by MSU crystals [24]. It is of note that the neurons which express the capsaicin sensor TRPV1 often co-express TRPA1 [8]. However, the non-neuronal TRPA1 expression and function has been clearly established in many cell types such as keratinocytes, synoviocytes and endothelium [39] giving rise to a possibility that MSU crystal-induced inflammation is also linked to the non-neuronal TRPA1 activation. Descriptively, activation of TRPA1 in vascular endothelium in addition to vascular sensory neurons is capable of causing edema through vasodilatation and increased vascular permeability [40]. Also, increase in intracellular Ca^{2+} concentration via activated TRPA1 ion channel may regulate the expression and release of inflammatory factors in both inflammatory and resident tissue cells as discussed above [35–37]. In our study we provide data originating from animal models focusing on tissue and organism level results of TRPA1 activation following the application of MSU crystals indicating that the responses are functional *in vivo* and not restricted to experimental *in vitro* conditions. Based on these results it is not, however, possible to define the cell types responsible for TRPA1 mediated responses in MSU crystal-induced inflammation yet it is likely that TRPA1 convoyed pro-inflammatory responses are due to the interaction between different neuronal and non-neuronal cell types.

In addition to the mechanisms described above, a more direct interaction between TRPA1 and IL-1β in MSU crystal-induced inflammation is possible, as TRPA1 activation has been reported to enhance IL-1β production in keratinocytes [33], and a similar effect was found in the present study in the air-pouch model. On the other hand, IL-1β has been found to enhance TRPA1 expression in human synovial cells especially in hypoxic conditions [41]. Although several potential mechanisms do exist, the exact means how TRPA1 exacerbates the MSU crystal-induced gouty inflammation cannot be conclusively defined by the present results but warrants further studies.

Currently, treatment of gout flare includes systemic or local corticosteroids, non-steroidal anti-inflammatory drugs and colchicine [1] but there is a real need for novel treatment modalities, especially in resistant cases. The present results propose TRPA1 as an attractive novel anti-inflammatory and analgesic drug target to treat acute gouty arthritis.

Conclusions

TRPA1 was found to mediate MSU crystal-induced inflammation and pain in three different experimental models supporting the role of TRPA1 as a potential mediator and a drug target in gout flare.

Supporting Information

S1 Table. Monosodium urate (MSU) crystals induced accumulation of inflammatory cytokines and cells into synovial joint mimicking subcutaneous air-pouch in the mouse. The

amounts of accumulated cells and of proinflammatory cytokines monocyte chemotactic protein-1 (MCP-1), interleukin-6 (IL-6), interleukin-1β (IL-1β), myeloperoxidase (MPO), macrophage inflammatory protein-1α (MIP-1α) and macrophage inflammatory protein-2 (MIP-2) measured in the synovial joint resembling subcutaneous air-pouch inflammation model in the mouse. The studied mice were injected into the air-pouch with 3 mg of monosodium urate (MSU) crystals in 1 ml of endotoxin free phosphate buffered saline (PBS) or with 1 ml of PBS only. The exudate was harvested 6h after the injection and cells were counted using hemocytometer and the cytokines were analysed using ELISA. Results are displayed as total amount of cells or cytokines per air-pouch. The results are expressed as mean ± SEM, n = 5–8, ∗ = $p<0.05$, ∗∗ = $p<0.01$, ∗∗∗ = $p<0.001$.
(PDF)

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Author Contributions

Conceived and designed the experiments: LJM MH RN EM. Performed the experiments: LJM MH. Analyzed the data: LJM LL. Contributed reagents/materials/analysis tools: LJM MH LL RMN EM. Wrote the paper: LJM MH LL RMN EM.

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

L.J. Moilanen, M. Hämäläinen, E. Nummenmaa, P. Ilmarinen, K. Vuolteenaho, R.M. Nieminen, L. Lehtimäki, E. Moilanen*

The Immunopharmacology Research Group, University of Tampere School of Medicine and Tampere University Hospital, Tampere, Finland

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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

* Address correspondence and reprint requests to: E. Moilanen, The Immunopharmacology Research Group, University of Tampere School of Medicine, 33014 Tampere, Finland.

E-mail addresses: lauri.j.moilanen@uta.fi (L.J. Moilanen), mari.j.hamalainen@uta.fi (M. Hämäläinen), elina.nummenmaa@uta.fi (E. Nummenmaa), pinja.ilmarinen@uta.fi (P. Ilmarinen), katriina.vuolteenaho@uta.fi (K. Vuolteenaho), riina.m.nieminen@uta.fi (R.M. Nieminen), lauri.lehtimaki@uta.fi (L. Lehtimäki), eeva.moilanen@uta.fi (E. Moilanen).

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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)].

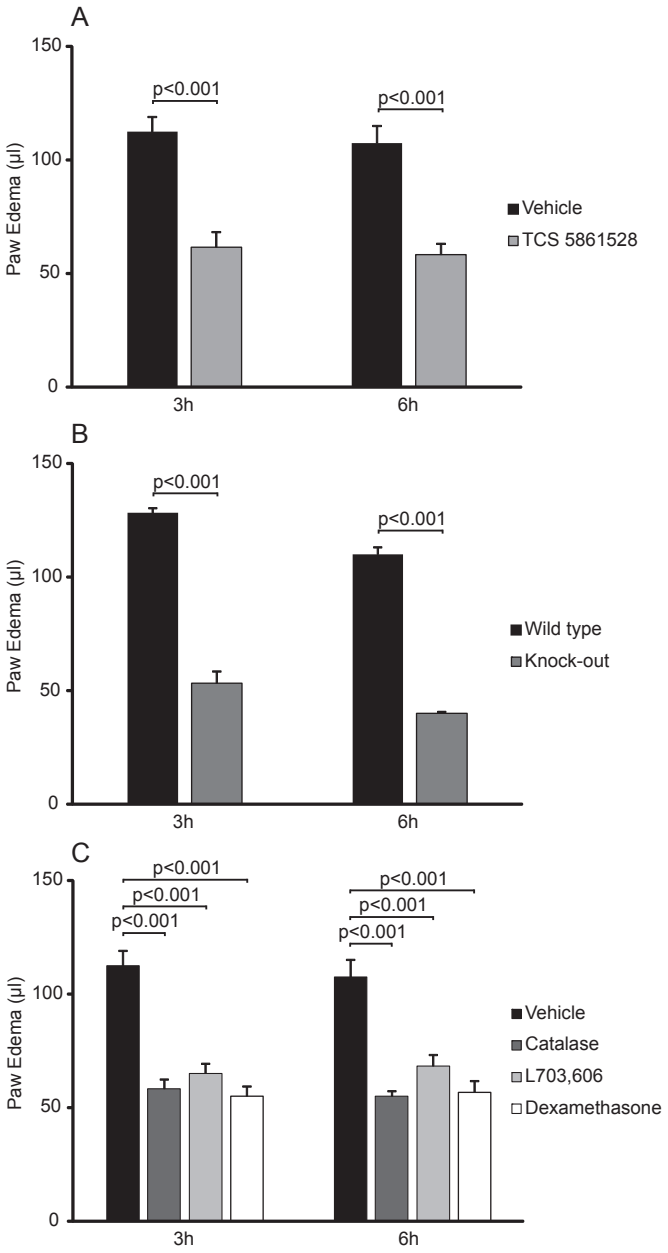

Fig. 1. MIA-induced acute paw edema was inhibited by both genetic depletion and pharmacological inhibition of TRPA1 as well as with treatment with the hydrogen peroxide detoxifying enzyme catalase and also the neurokinin 1 receptor antagonist. MIA induced an acute inflammatory edema when injected into 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).

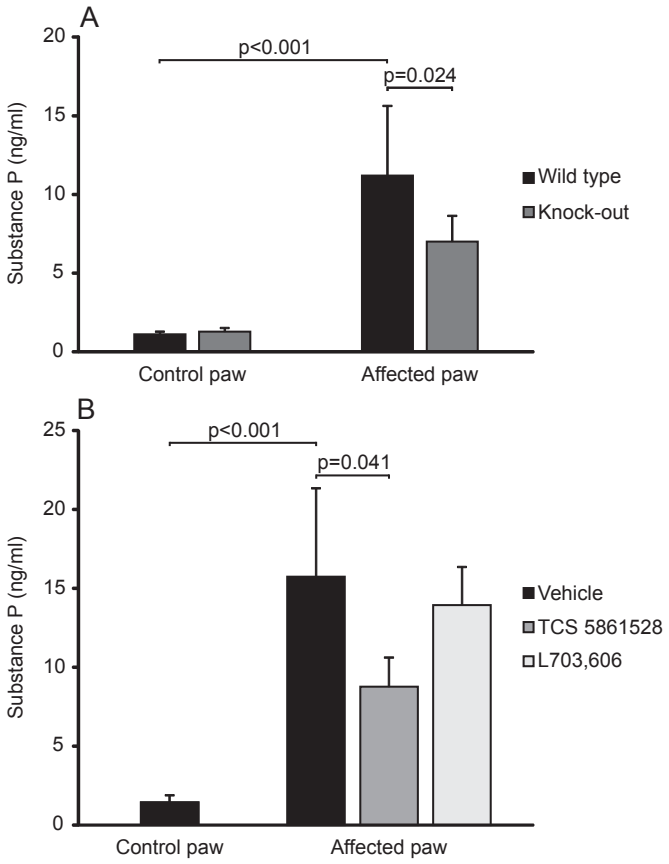

Fig. 2. MIA induced the release of the neuropeptide substance P into the inflamed paw; this effect was inhibited by both genetic depletion and pharmacological inhibition of TRPA1 but not by treatment with the neurokinin 1 receptor antagonist. Substance P concentrations were elevated in the mouse paw 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

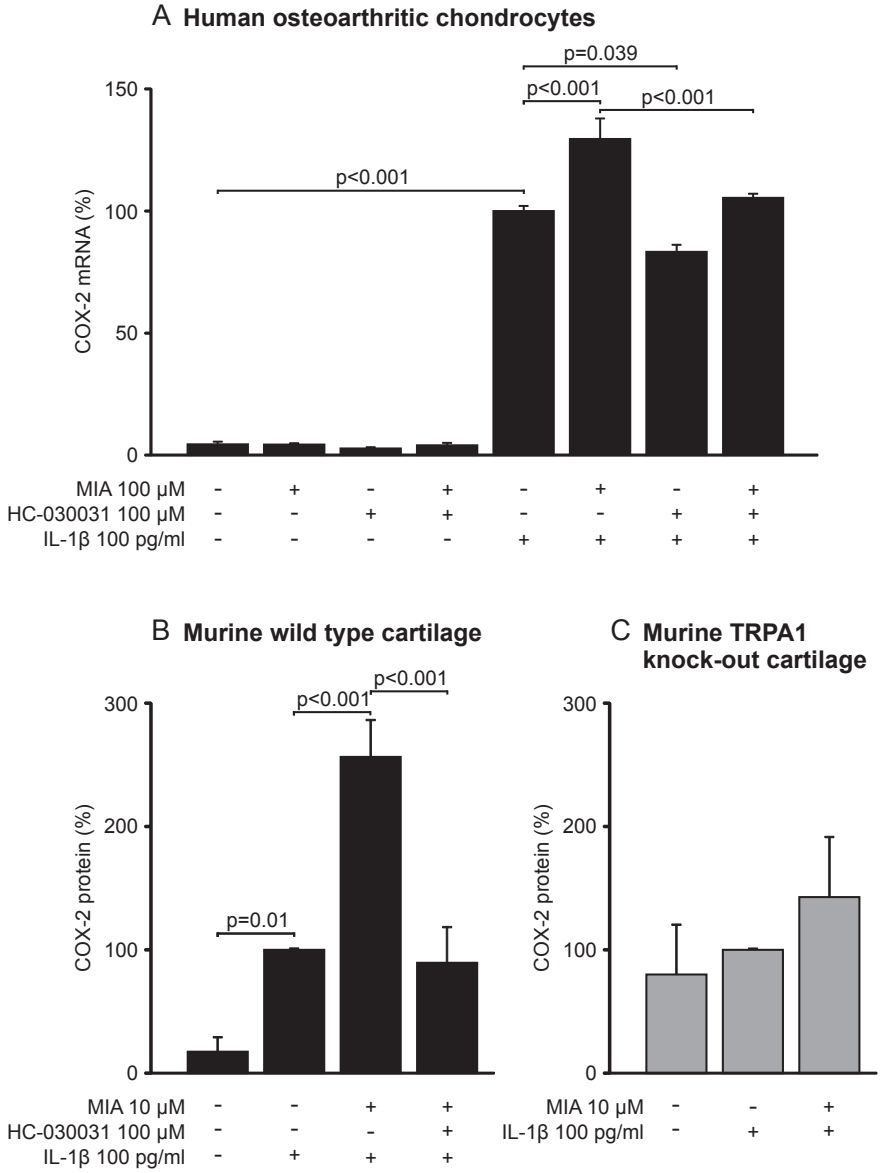

Fig. 3. MIA-induced COX-2 expression was reduced by pharmacological inhibition and genetic depletion of TRPA1. In primary cultures of human osteoarthritic chondrocytes, treatment with the TRPA1 antagonist HC-030031 reduced the expression of the proinflammatory gene COX-2 induced by co-stimulation with 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

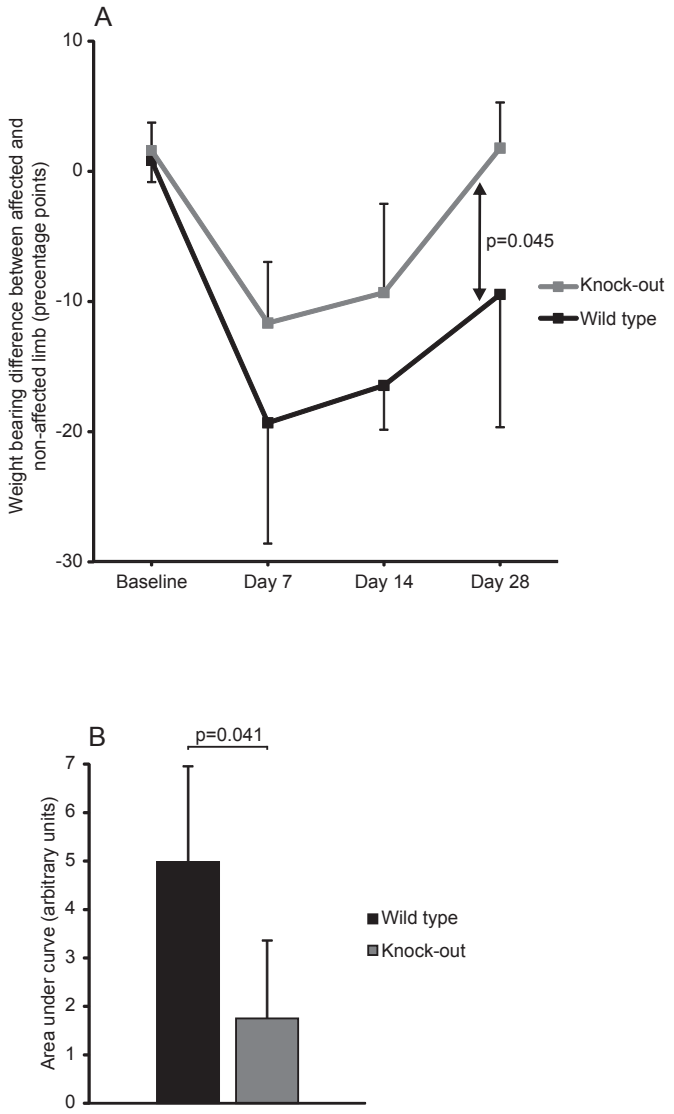

Fig. 4. TRPA1 deficient mice exhibited an attenuated response to MIA in the weight-bearing test indicative of alleviated joint pain. When MIA was injected into one knee joint, wild type mice exhibited a reduction in spontaneous weight-bearing on the affected limb indicative of joint pain. Interestingly, 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

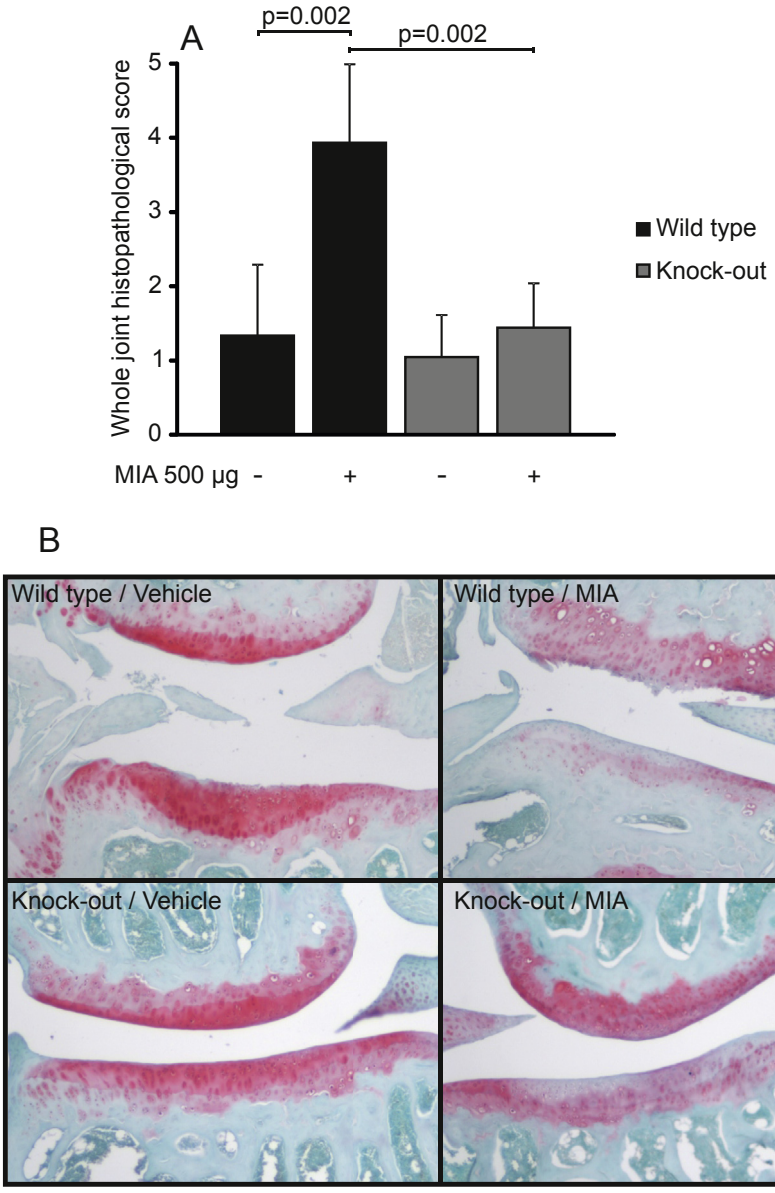

Fig. 5. TRPA1 deficient mice developed less severe cartilage changes in the knee joint following MIA injection. When MIA was injected into the knee joint, wild type mice developed more severe cartilage changes than TRPA1 deficient (knock-out) mice. Twenty-eight days after intra-articular injection of 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

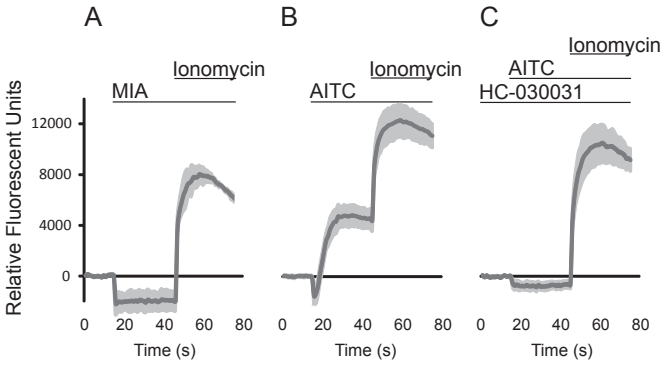

Fig. 6. MIA did not induce TRPA1 mediated Ca^{2+}-influx into HEK 293 cells transfected with TRPA1. MIA did not evoke TRPA1-mediated Ca^{2+}-influx (A) whereas the known TRPA1 agonist AITC induced a robust Ca^{2+}-influx (B) which was inhibited by pre-treatment with the selective TRPA1 antagonist HC-030031 (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

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Short communication

Pinosylvin Inhibits TRPA1-Induced Calcium Influx *In Vitro* and TRPA1-Mediated Acute Paw Inflammation *In Vivo*

Lauri J. Moilanen[1], Mari Hämäläinen[1], Lauri Lehtimäki[2], Riina M. Nieminen[1], Katsuhiko Muraki[3] and Eeva Moilanen[1]

[1]The Immunopharmacology Research Group, University of Tampere School of Medicine and Tampere University Hospital, Tampere, Finland, [2]Department of Respiratory Medicine, University of Tampere School of Medicine and Allergy Centre, Tampere University Hospital, Tampere, Finland and [3]Laboratory of Cellular Pharmacology, School of Pharmacy, Aichi-Gakuin University, Nagoya, Japan

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Pinosylvin (3,5-dihydroxy-*trans*-stilbene) is a stilbenoid polyphenol structurally related to resveratrol, and it is found in heartwood, knot and bark of *Pinus* species. Pinosylvin has been shown to possess anti-inflammatory and cancer-chemopreventive properties as well as being an antioxidant preventing oxidative stress [1–4].

Transient receptor potential ankyrin 1 (TRPA1) is an ion channel expressed in the terminal ends of sensory neurons throughout the body. It functions as a chemosensor for potentially harmful environmental substances, and it is also involved in pathophysiological conditions such as pain, hyperalgesia and inflammation [5–8]. Curiously, pharmacological inhibition of TRPA1 has shown positive results in animal models of inflammatory diseases such as asthma [9], colitis [10] and gout [11,12]. However, rather few compounds are known to antagonize TRPA1: there is a clear need for new substances with the ability to block this ion channel. Interestingly, resveratrol has shown putative activity as a TRPA1 antagonist [13]. Therefore, we were interested in studying the effect of pinosylvin on TRPA1 *in vitro* by measuring TRPA1-mediated Ca^{2+} influx and membrane currents, and by investigating *in vivo* the effect of pinosylvin on TRPA1-induced acute inflammation.

Materials and Methods

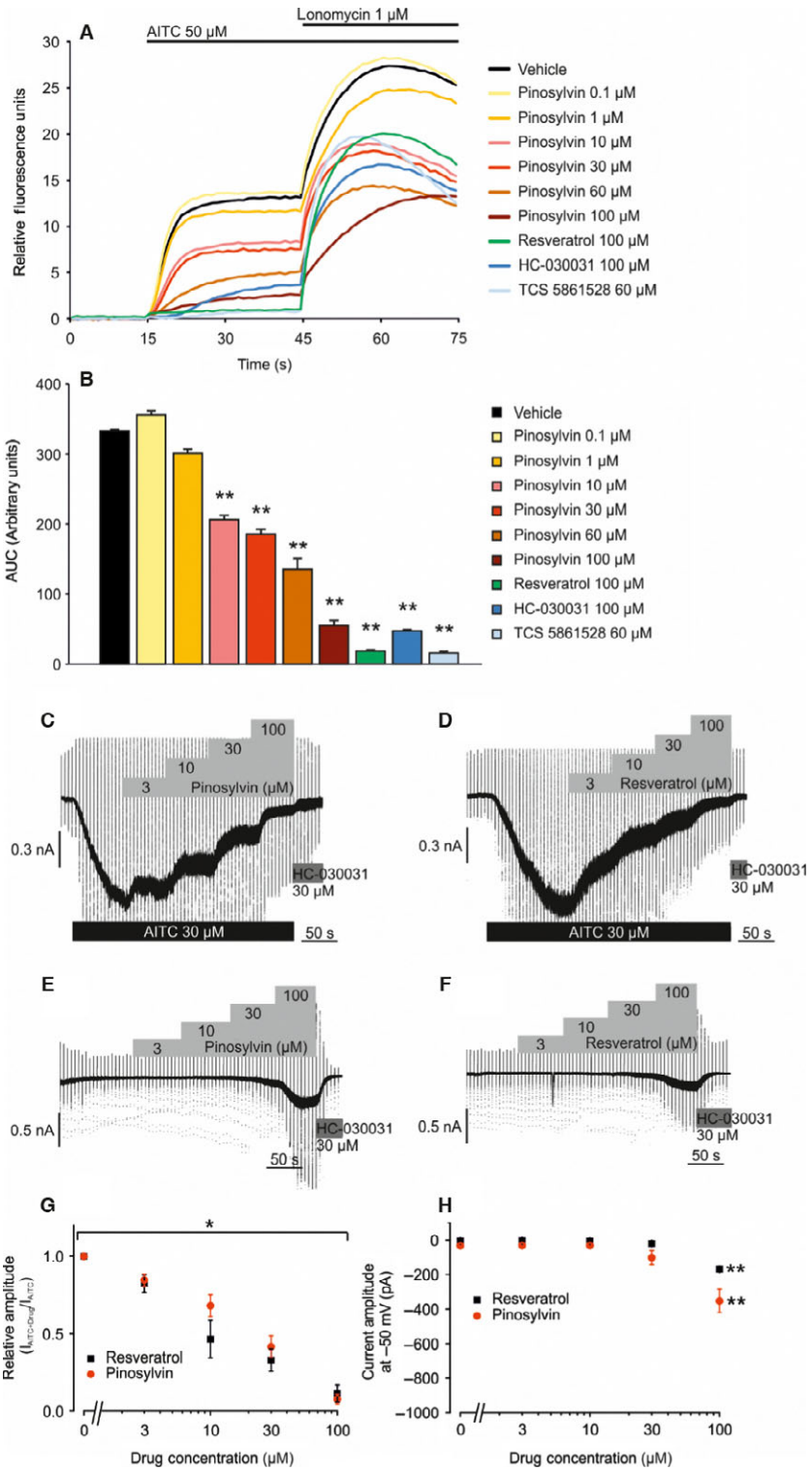
The Fluo-3-AM assay was used to measure the TRPA1-mediated increase in the intracellular Ca^{2+} concentration in HEK293 cells transiently transfected with a plasmid encoding hTRPA1 (pCMV6-XL4; Origene, Rockville, MD, USA) as described previously [7]. In brief, the cells were loaded with 4 μM Fluo-3-acetoxymethyl ester and 0.08% Pluronic F-127® in Hanks' balanced salt solution containing bovine serum albumin (1 mg/ml), 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 a Victor3 1420 multi-label counter (Perkin Elmer, Waltham, MA, USA) at excitation/emission wavelengths of 485/535 nm. The cells were first pre-incubated with the vehicle or with pinosylvin (0.1–100 μM), resveratrol (100 μM) or the TRPA1 antagonists HC-030031 (100 μM) or TCS 5861528 (60 μM) dissolved in dimethyl sulphoxide (final concentration 0.1%) for 30 min. at +37°C. Thereafter, the TRPA1 agonist allyl isothiocyanate (AITC, 50 μM) was added and the measurements were continued for 30 sec. after which a robust Ca^{2+} influx was induced by application of the control ionophore compound ionomycin (1 μM).

Whole-cell patch clamp experiments were carried out as described previously [8] to record TRPA1-mediated membrane currents in HEK293 cells transiently transfected with a plasmid encoding hTRPA1 (pIRES2-AcGFP1; Takara, Tokyo, Japan). The resistance of electrodes was 3–5 MΩ when filled with the pipette solution (Cs-aspartate 110 mM, CsCl 30 mM, $MgCl_2$ 1 mM, HEPES 10 mM, EGTA 10 mM, $CaCl_2$ 6.25 mM, ATP-Na_2 2 mM, adjusted to pH 7.2 with CsOH). Membrane currents and voltage signals were digitized into a computer using an analogue–digital converter (PCI6229; National Instruments Japan Corporation, Tokyo, Japan). Data acquisition and analysis of whole-cell currents were performed using WinEDR V3.38 developed by Dr. John Dempster (University of Strathclyde, UK). The liquid junction potential between the pipette and bath solutions (−10 mV) was corrected. A ramp voltage protocol from −150 mV to +100 mV of 400 ms was applied every 5 sec. from a holding potential of −50 mV. A HEPES-buffered bathing solution (NaCl 137 mM, KCl 5.9 mM, CsCl 10 mM, $MgCl_2$ 1.2 mM, glucose 14 mM, HEPES 10 mM, adjusted to pH 7.4 with NaOH) was used. Extra- and intracellular Ca^{2+} was omitted and adjusted to 0.3 μM in the bathing and pipette solution, respectively, to maintain TRPA1 channel activity. All experiments were performed at 25 ± 1°C, and the studied drugs, resveratrol (3-100 μM), pinosylvin (3–100 μM) and HC-030031 (30 μM), were dissolved in dimethyl sulphoxide (final concentration ≤ 0.13%).

Male C57BL/6N mice (Scanbur Research A/S, Karlslunde, Denmark) were used in the *in vivo* experiments. The mice were housed under standard conditions (12:12-hr light:dark cycle, 22 ± 1°C) with food and water provided freely. 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 injection of medetomidine (0.5 mg/kg, Domitor®; Orion Oyj, Espoo, Finland) and ketamine (75 mg/kg, Ketalar®; Pfizer Oy Animal Health, Helsinki, Finland) was used for anaesthesia. Animals were sacrificed after experiments by carbon monoxide followed by cranial dislocation.

Author for correspondence: Eeva Moilanen, The Immunopharmacology Research Group, University of Tampere School of Medicine, 33014 Tampere, Finland (e-mail eeva.moilanen@uta.fi).

Inflammatory paw oedema was induced by injecting the TRPA1 agonist AITC (10 mM) in 50 µl of sterile endotoxin-free PBS into the hind paw of anaesthetized mice. The contralateral paw was injected with the corresponding volume of the vehicle and developed no measurable oedema. The mice were dosed 1 hr prior to the injection of AITC with pinosylvin (10 mg/kg in 250 µl of PBS intraperitoneally), resveratrol (10 mg/kg in 250 µl of PBS intraperitoneally), TRPA1 antagonist TCS 5861528 (10 mg/kg in 250 µl of a mixture of polyethylene glycol 300 (50%), propylene glycol (40%) and glucosteril polyethylene glycol (10%) orally) or with the vehicle (250 µl of PBS intraperitoneally). The paw volume was measured up to 6 hr with a plethysmometer (Ugo Basile, Comerio, Italy) and compared to the baseline value. After the mice had been killed, the inflamed subcutaneous paw tissue injected with AITC and the contralateral paw tissue was collected into a buffer containing Tris (50 mM, pH 7.4), NaCl (150 mM), 0.5% Triton X and protease and phosphatase inhibitors



phenylmethylsulphonyl 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 ice-cold lysis buffer for 20 min. at +4°C in constant shaking. The samples were centrifuged (10,000 × *g*, 10 min.), and the supernatant was collected and measured for interleukin-6 (IL-6) by ELISA (R&D Systems Europe Ltd., Abingdon, UK).

Reagents were purchased from Sigma Chemical Co., St. Louis, MO, USA, unless otherwise indicated. Data were analysed with SPSS version 17.0 for Windows (SPSS Inc, Chicago, IL, USA) or with Origin 9.10J (OriginLab Corp, Northampton, MA, USA) software using two-way (fig. 1G) or one-way ANOVA with Bonferroni's or Tukey's multiple comparison test.

Results

Pinosylvin inhibited TRPA1-mediated Ca^{2+} influx in a dose-dependent manner when measured by the Fluo-3-AM assay in TRPA1-transfected HEK293 cells as shown in fig. 1A and B (IC50 26.5 μM and HillSlope -0.77). Two established TRPA1 antagonists, HC-030031 and TCS 5861528, and another stilbenoid, resveratrol (which has previously been shown to inhibit TRPA1 [13]), were used as control compounds. The inhibitory effect of pinosylvin on TRPA1-mediated Ca^{2+} influx was confirmed in patch clamp studies: treatment with pinosylvin reversed the effect of the TRPA1 agonist AITC on membrane currents as seen in fig. 1C (IC50 16.7 μM and HillSlope -1.17), and the effect was comparable to that of resveratrol as seen in fig. 1D (IC50 12.9 μM and HillSlope -0.99). Curiously, as seen in fig. 1E and F, at a high concentration (100 μM), pinosylvin and resveratrol also showed a minor activating effect on TRPA1. As predicted, AITC or pinosylvin did not evoke measurable responses in non-transfected HEK293 cells in either of the assays (data not shown).

The results seen *in vitro* on the effect of pinosylvin as a TRPA1 antagonist were also tested *in vivo* in the AITC-induced acute inflammation. In these experiments, the mice treated with pinosylvin exhibited a significantly attenuated acute oedema in response to AITC (fig. 2A); the effect was comparable to that of the known TRPA1 antagonist TCS 5861528 and resveratrol. Furthermore, the analysis of the inflamed paw tissue indicated that the increased production of the pro-inflammatory cytokine IL-6 was blunted by the treatment with pinosylvin (fig. 2B) and correspondingly with TCS 5861528 or resveratrol. Together, these results on AITC-induced acute paw inflammation revealed the anti-inflammatory potential of pinosylvin in TRPA1-mediated inflammation *in vivo*.

Discussion

The present results showed for the first time the clear inhibitory effect of pinosylvin on TRPA1 *in vitro* and *in vivo*. Both Ca^{2+} influx and membrane currents assays, i.e. Fluo-3-AM assay and patch clamping, confirmed the dose-dependent inhibitory effect of pinosylvin on AITC-induced TRPA1-mediated responses. The positive results were duplicated in a model of acute paw inflammation triggered by the direct TRPA1 agonist AITC, that is mice treated with pinosylvin displayed a diminished formation of paw oedema and attenuated production of the powerful pro-inflammatory cytokine IL-6 at the site of the inflammation.

Resveratrol and pinosylvin are structurally related naturally occurring stilbenoids. Resveratrol has been claimed to have antioxidant, anti-inflammatory, antiproliferative and chemoprotective properties, with inhibition of TRPA1 being recently postulated as a novel potential mechanisms of action [13,14]. Pinosylvin has also been reported to possess some of these functions, especially anti-inflammatory effects [2,4], but the effect of pinosylvin on TRPA1 had not been investigated earlier. The present study introduces pinosylvin as a novel TRPA1 antagonist and both confirms and extends the inhibitory effect of resveratrol on TRPA1, until now only reported in a single study [13]. The detailed mechanism of action of resveratrol on TRPA1 has not been elucidated, but resveratrol shows properties indicative of suppression of AITC-induced maximal Ca^{2+} influx rather than increasing the concentration of AITC needed for full response [13]. The present results are also not able to reveal the exact molecular inhibitory mechanism of pinosylvin on TRPA1. AITC is known to activate TRPA1 rapidly by covalent binding to intracellular cysteines on the ion channel [15]. Based on our data from Ca^{2+} influx imaging in Fluo-3-AM assay and patch clamping, it seems that

Fig. 1. Pinosylvin inhibited TRPA1 activation in a dose-dependent manner when measured by Ca^{2+} influx and membrane current assays, that is Fluo-3-AM assay and patch clamping, in HEK293 cells transfected with a plasmid encoding TRPA1 and activated with the TRPA1 agonist, allyl isothiocyanate (AITC). The effects of pinosylvin were comparable to those of the established selective TRPA1 antagonists HC-030031 and TCS 5861528, and to resveratrol, which were used as control compounds. (A) and (B) show the results of the Fluo-3-AM assay. In (A), the curves display relative fluorescent units which reflect the intracellular Ca^{2+} concentration, and in (B), area under curve values were calculated between timepoints of 15 and 45 sec. during which the cells were exposed to AITC. The Fluo-3-AM assay was carried out as described in Materials and Methods. Briefly, the cells were loaded with Fluo-3-AM, and the intracellular Ca^{2+} concentration was assessed in a Victor3 multi-label counter at excitation/emission wavelengths of 485/535 nm. The cells were pre-incubated with the studied compound for 30 min. at +37°C before the commencement of the measurements. The basal fluorescence was first recorded for 15 sec., and thereafter, the TRPA1 agonist AITC (50 μM) was added and the measurement was continued for 30 sec. after which the control ionophore compound ionomycin (1 μM) was introduced to the cells. The results are normalized against background and expressed as mean (A) and mean + S.E.M. (B), n = 6 (A and B), $^{**}p < 0.01$ (B). (C–G) show the results of the patch clamp experiments. In (C) and (D), the trace displays the change of inward currents at −50 mV which was evoked by pinosylvin and resveratrol in the presence of AITC (30 μM), and in (G), the relative amplitude ($I_{AITC\text{-}Drug}/I_{AITC}$) was calculated between the current size before (I_{AITC}) and after application of each drug ($I_{AITC\text{-}Drug}$). In (E) and (F), the trace displays the change of inward currents at −50 mV which was evoked by pinosylvin and resveratrol without AITC, and in (H), each current amplitude (pA) was summarized. Details of patch clamp experiments are described in Materials and Methods. The results in (G) and (H) are expressed as mean ± S.E.M., n = 5 (G) and n = 4 (H), $^{*}p < 0.05$ and $^{**}p < 0.01$.

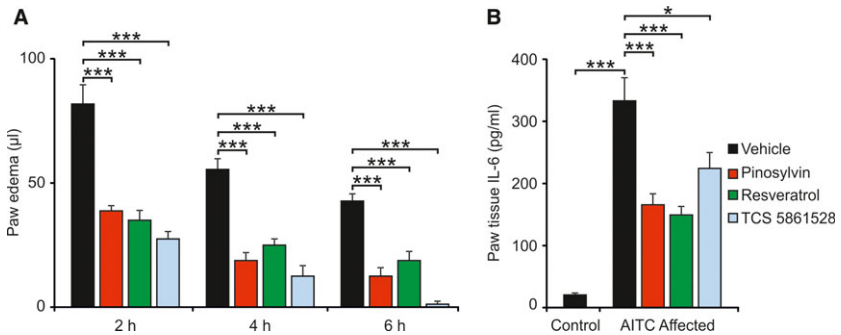


Fig. 2. Pinosylvin exerted an inhibitory effect on TRPA1-induced acute inflammation. TRPA1 agonist allyl isothiocyanate (AITC) triggered an acute inflammatory response when injected into the mouse hind paw, and the extent of the inflammatory oedema was significantly attenuated in mice treated with pinosylvin (10 mg/kg), resveratrol (10 mg/kg) or the TRPA1 antagonist TCS 5861528 (10 mg/kg) (A). When measured 6 hr after the commencement of the experiment, the paw tissue injected with AITC showed greatly increased levels of the pro-inflammatory cytokine interleukin-6 (IL-6); these elevated IL-6 levels were reduced by treatment with pinosylvin, resveratrol or TCS 5861528 (B). To assess paw oedema, the paw volume was measured with a plethysmometer before and 2, 4 and 6 hr after the paw had been injected with AITC (10 mM dissolved in 50 µl of sterile endotoxin-free PBS). The contralateral control paw injected with the solvent developed no measurable oedema. Paw oedema is expressed as the volume change from the baseline value. After 6 hr, the mice were sacrificed and the inflamed and contralateral paw tissues were collected and measured for IL-6 by ELISA. The results are expressed as mean + S.E.M.. n = 6, $*p < 0.05$, $***p < 0.001$.

pinosylvin evokes a dose-dependent and rapid inhibition of TRPA1; and pinosylvin exerted its effect either when applied prior to or after the AITC. As pinosylvin and resveratrol have been identified to perpetrate several effects some of which are anti-inflammatory, it is likely that resveratrol and pinosylvin are not selective TRPA1 inhibitors but TRPA1 antagonism is a significant mechanism contributing to their therapeutic effects. Moreover, stilbenoids could serve as a structural groundwork for future development of TRPA1 inhibiting drugs.

In addition to the clear dose-dependent inhibition of TRPA1, a high concentration of resveratrol and pinosylvin was surprisingly found to induce a minor TRPA1-mediated inward current referring to channel activation. Interestingly, some other compounds have also been noted to have bimodal effects on TRPA1. Curiously, some TRPA1 inhibiting oxime derivatives have been found to have a similar biphasic activity as pinosylvin and resveratrol in the present study, possibly because of covalent modification of the ion channel [16]. On the other hand, substances such as cinnamaldehyde, camphor and apomorphine have an opposite bimodal effect: at low concentrations, they elicit a dose-dependent activation of TRPA1, but at high concentrations, the effect is inhibitory [5]. In the case of stilbenoids, it is possible that different optical enantiomers may have opposite pharmacological actions on TRPA1. Also, as stilbenoids possess relatively reactive double bonds in their structures, resveratrol and pinosylvin at high concentrations form covalent bonds with TRPA1 and thereby act as weak TRPA1 agonists.

TRPA1 has been proven to be crucial in the development of many pathological conditions related to acute pain, inflammation and hyperalgesia, and thus, its inhibition may be beneficial in many inflammatory conditions as shown in experimental carrageenan-induced paw inflammation [7], allergic contact dermatitis [17], asthma [9], gout [11,12] and colitis [10]. Furthermore, TRPA1 has been shown to be important also in human beings; a genetic gain-of-function variant of TRPA1 has been shown to increase pain sensation [18] and odour perception [19] and direct activation of TRPA1 increased mucus secretion [19] and evoked local hyperalgesia and vasodilation [20]. Because of the plethora of encouraging results on the pharmacological inhibition or genetic depletion of TRPA1, it appears to be an appealing drug target. Interestingly, the first phase II clinical trial with a TRPA1 antagonist is ongoing and the latest press release from September 2014 by Glenmark Pharmaceuticals Ltd. reported positive data in patients with painful diabetic neuropathy [21].

Taken together, this short communication highlights the ability of pinosylvin to inhibit the action of TRPA1 *in vitro* and *in vivo* and proposes that stilbenoids could provide a useful source of novel TRPA1 antagonists.

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