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PARTICIPAÇÃO DO RECEPTOR TRPA1 EM MODELOS DE ATAQUE AGUDO DE GOTA EM ROEDORES

TESE DE DOUTORADO

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Santa Maria, RS, Brasil,
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PARTICIPAÇÃO DO RECEPTOR TRPA1 EM MODELOS DE ATAQUE AGUDO DE GOTA EM ROEDORES

Gabriela Trevisan dos Santos

Tese apresentada ao Curso de Doutorado do Programa de Pós-Graduação em Ciências Biológicas: Bioquímica Toxicológica da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para a obtenção do grau de
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PARTICIPAÇÃO DO RECEPTOR TRPA1 EM MODELOS DE ATAQUE AGUDO DE GOTA EM ROEDORES

elaborada por

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como requisito parcial para obtenção do grau de
Doutor em Bioquímica Toxicológica

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Santa Maria, 21 de outubro de 2013.

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“Se as coisas são inatingíveis... ora!
Não é motivo para não querê-las...
Que tristes os caminhos, se não fora
A presença distante das estrelas!”

Mario Quintana em “Espelho Mágico”.

RESUMO

Tese de Doutorado
Programa de Pós-graduação em Ciências Biológicas: Bioquímica Toxicológica
Universidade Federal de Santa Maria

PARTICIPAÇÃO DO RECEPTOR TRPA1 EM MODELOS DE ATAQUE AGUDO DE GOTA EM ROEDORES

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ORIENTADOR: JULIANO FERREIRA
Data e Local da Defesa: Santa Maria, 21 de outubro de 2013.

A gota é uma forma prevalente de artrite inflamatória que reduz a qualidade de vida dos pacientes. Os ataques agudos de gota produzem dor articular grave e inflamação que são associadas à produção de estresse oxidativo. Esta patologia é provocada pela deposição de cristais de urato monossódico (MSU), mas os mecanismos relacionados à dor observada nos ataques agudos de gota ainda são pouco esclarecidos. O receptor de potencial transitório anquirina 1 (TRPA1) é um sensor para compostos oxidantes, como o peróxido de hidrogênio (H_2O_2), encontrado em fibras sensoriais peptidérgicas e este está relacionado ao desenvolvimento de dor inflamatória. O objetivo deste estudo foi avaliar o papel do receptor TRPA1 em dois modelos de inflamação e nocicepção induzidos pela administração de cristais de MSU em ratos e camundongos. Observamos que o antagonismo do receptor TRPA1 (HC-030031 ou cânfora), a deleção genética deste canal, ou ainda a indução de dessensibilização dos neurônios sensoriais que expressam os receptores TRP pelo tratamento com capsaicina reduziram marcantemente a nocicepção e edema induzido pela administração intraplantar (i.pl.) ou intra-articular (i.a.) dos cristais de MSU. Além destes efeitos neurogênicos a administração de MSU aumentou o conteúdo de H_2O_2 nos tecidos injetados, um efeito que foi bloqueado pela enzima catalase, e também aumentou a imunoreatividade para o receptor TRPA1 no nervo ciático e no tecido sinovial, e também os níveis do peptídeo relacionado ao gene da calcitonina (CGRP) no tecido sinovial. A administração de H_2O_2 por via i.pl. ou i.a. induziu efeitos semelhantes àqueles induzidos pela administração de MSU, e estes foram reduzidos pela administração de antagonistas TRPA1. Ainda, o bloqueio do receptor TRPA1 reduziu a infiltração de neutrófilos e a produção de interleucina 1β induzidas pela administração de cristais de MSU. Em conclusão, os nossos resultados sugerem que a administração dos cristais de MSU é capaz de aumentar a produção de H_2O_2 que então poderia estimular o receptor TRPA1 expresso em neurônios sensoriais causando nocicepção e inflamação dos tecidos. Dessa maneira, o canal TRPA1 poderia ser explorado como um alvo em potencial para o tratamento dos ataques agudos de gota.

Palavras-chave: Gota. Dor. TRPA1. Peróxido de hidrogênio. IL-1β. CGRP.

ABSTRACT

PhD Thesis
Graduate Course in Biological Sciences: Toxicological Biochemistry
Federal University of Santa Maria

Participation of TRPA1 receptor in acute gout attack models in rodents

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ADVISOR: JULIANO FERREIRA
Date and Place of the Defense: Santa Maria, October, 21st, 2013.

Gout is a prevalent form of inflammatory arthritis, which leads to patients' poor quality of life. Acute gout attacks produce severe joint pain and inflammation associated with oxidative stress induction. This pathology is provoked by the accumulation of monosodium urate (MSU) crystals, but the underlying pain mechanisms in acute gout attacks are still poorly understood. The transient potential receptor ankyrin 1 (TRPA1) is a sensor for endogenous oxidant compounds, such as hydrogen peroxide (H_2O_2), found in peptidergic sensory fibers associated to inflammatory pain. The goal of this study was to explore the TRPA1 role in two models of monosodium urate (MSU) crystals-induced inflammation and nociception in rats and mice. We found that TRPA1 antagonism (HC-030031 or camphor), TRPA1 gene deletion or defunctionalization by capsaicin pretreatment of peptidergic TRP-expressing primary sensory neurons markedly decreased MSU-induced nociception and edema after intraplantar (i.pl.) or intra-articular (i.a.) injection. In addition to these neurogenic effects, MSU increased H_2O_2 levels in the injected tissue, an effect that was abolished by the H_2O_2-detoxifing, catalase enzyme. TRPA1 immunoreactivity in sciatic nerve and the levels of calcitonin gene related peptide (CGRP) in the synovial tissue were also increased by MSU. H_2O_2 i.pl. or i.a. injection mimicked MSU, causing nociception and edema prevented by TRPA1 antagonism. Moreover, TRPA1 blockage abrogated the increase in neutrophil infiltration and interleukin-1β elicited by MSU. Our results suggest that MSU-injection increases tissue H_2O_2 thereby stimulating TRPA1 on sensory nerve endings to produce inflammation and nociception. Thus, TRPA1 may be explored as a valuable target in acute gout management.

Keywords: Gout. Pain. TRPA1. Hydrogen peroxide. IL-1β. CGRP.

LISTA DE FIGURAS

Figura 1 - Anatomia dos mecanismos associados à transmissão de estímulos dolorosos. Adaptado de Basbaum et al. (2009). 14
Figura 2 - Mecanismos associados à inflamação aguda observada na gota. Adaptado de Terkeltaub (2010). 25
Figura 3 - Representação espacial da estrutura das diferentes famílias de receptores TRP. Adaptado de Montell (2005). 32
Figura 4 - Mecanismos de ativação e sensibilização do receptor TRPA1. Adaptado de Bautista et al. (2013). 38
Figura 5 - Participação central do receptor TRPA1, mediada pela ativação por H_2O_2, nos mecanismos envolvidos na nocicepção e inflamação mediada pela injeção de cristais de MSU em roedores. (1) Primeiramente a fagocitose dos cristais de MSU pelos sinoviócitos (tipo macrófago tipo A) poderia levar a produção de H_2O_2, (2) este levaria a ativação do receptor TRPA1 expresso em neurônios nociceptivos, (3) causando a liberação de neuropeptídeos vasoativos como o CGRP e SP e também a respostas dolorosas, (4) a estimulação de receptores para estes peptídeos expressos em células endoteliais poderia levar a (4) infiltração de neutrófilos e então (5) a ativação do NALP3 e produção de IL-1β por estas células inflamatórias. 109

LISTA DE ABREVIATURAS

AINES	Anti-inflamatórios não esteroidais
AITC	Isotiocianato de alila
CFA	Adjuvante completo de Freund
CGRP	Peptídeo relacionado ao gene da calcitonina
DAG	Diacilglicerol
EULAR	Liga Européia Contra o Reumatismo
H_2O_2	Peróxido de hidrogênio
4-HNE	4-hidroxinonenal
IASP	Associação Internacional para o Estudo da Dor (IASP)
IL	Interleucina
IP3	Inositol-1,4,5-trifosfato
GN	Gânglio nodoso
GPCR	Receptores acoplados à proteína G
GRD	Gânglio da raiz dorsal
GT	Gânglio do trigêmio
GV	Gânglio vagal
MSU	Urato monossódico
PIP2	fosfatidil-inositol-4,5-bifosfato
PLC	Fosfolipase C
PKA	Proteína cinase A
PKC	Proteína cinase C
ROS	Espécies reativas de oxigênio
RTX	Resiniferatoxina
SP	Substância P
TLR	Receptor do tipo Toll
TNF-α	Fator de necrose tumoral
TRPA1	Receptor de potencial transitório anquirina 1
TRPV1	Receptor de potencial transitório vanilóide 1

LISTA DE APÊNDICES

Apêndice A – Artigo publicado na revista Neuropharmacology: A novel, potent, oral active and safe antinociceptive pyrazole targeting kappa opioid receptors 124

SUMÁRIO

1 INTRODUÇÃO 11
1.1 Apresentação 11
1.2 Dor e nocicepção 11
1.3 Gota 15
1.3.1 Histórico 15
1.3.2. Epidemiologia 17
1.3.3. Manifestações clínicas da gota 18
1.3.4 Fisiopatologia da inflamação associada à artrite úrica 20
1.3.5 Diagnóstico e tratamento da gota 25
1.3.6 Dor relacionada à gota e impacto na qualidade de vida 29
1.4 Receptor TRPA1 31
1.4.1 Receptores de potencial transitório – TRP 31
1.4.2 Receptor de potencial transitório anquirina 1 – TRPA1 34
1.4.2.1 Aspectos gerais do receptor TRPA1 34
1.4.2.2 Participação do receptor TRPA1 em patologias dolorosas 38
2 OBJETIVOS 41
2.1 Objetivo Geral 41
2.2 Objetivos Específicos 41
3 ARTIGOS 42
3.1 Artigo 1 43
3.2 Artigo 2 79
4 DISCUSSÃO 101
5 CONCLUSÕES 108
REFERÊNCIAS BIBLIOGRÁFICAS 110
APÊNDICE 124

1 INTRODUÇÃO

1.1 Apresentação

No item **INTRODUÇÃO** consta uma revisão sucinta da literatura sobre os temas abordados nesta tese.

A metodologia realizada e os resultados obtidos que compõem esta tese estão apresentados sob a forma de artigos científicos que foram publicados ou enviados para publicação, os quais se encontram no item **ARTIGOS**. As seções Materiais e Métodos, Resultados, Discussão e Referências Bibliográficas encontram-se nos próprios artigos e representam a íntegra deste estudo.

Os itens **DISCUSSÃO**, **CONCLUSÕES** e **REFERÊNCIAS BIBLIOGRÁFICAS** encontram-se no final desta tese.

As **REFERÊNCIAS BIBLIOGRÁFICAS** referem-se somente as citações que aparecem nos itens Introdução e Discussão desta tese.

1.2 Dor e nocicepção

A dor é um dos sintomas clínicos mais comuns para a procura de cuidados médicos nos serviços primários de saúde e também para o uso de medicamentos. Porém, o tratamento adequado da dor ainda é considerado um desafio terapêutico, tanto em países desenvolvidos como em desenvolvimento, e é estimado que 80% da população mundial têm acesso limitado a medicamentos para tratamento da dor moderada a grave. Sendo assim, o tratamento ineficaz da dor reduz consideravelmente a qualidade de vida e poderia ser considerado como uma revogação de um direito humano (BRENNAN; CARR; COUSINS, 2007; COUSINS; LYNCH, 2011; KING; FRASER, 2013; LOHMAN; SCHLEIFER; AMON 2010).

A capacidade de detectar estímulos nocivos é essencial para a sobrevivência e bem-estar de um organismo. Porém, mesmo que a dor normalmente funcione

como uma resposta de alerta aos indivíduos, ela pode se tornar um sintoma debilitante como ocorre na dor crônica (WOOLF, 2010). Assim, a dor pode causar uma redução drástica na qualidade de vida dos pacientes e levar a implicações em diversos níveis como psicológico e emocional (podendo aumentar a incidência de doenças psiquiátricas como a depressão), social (diminuindo a capacidade de trabalho e podendo levar ao desemprego), fisiológico (causando o desenvolvimento de dor crônica e o aumento das complicações pós-operatórias), e também econômico (levando a redução da força de trabalho e aumento dos gastos com serviços de saúde e medicamentos). Dessa maneira, o tratamento efetivo da dor deve ser considerado como uma prioridade, principalmente em doenças associadas a dor crônica (BRENNAN; CARR; COUSINS, 2007; COUSINS; LYNCH, 2011; KING; FRASER, 2013; LOHMAN; SCHLEIFER; AMON, 2010).

A dor é definida pela Associação Internacional para o Estudo da Dor (IASP) como “uma experiência sensorial e emocional desagradável, associada a uma lesão tecidual real ou potencial, ou descrita em termos que sugerem tal lesão” (LOESER; TREEDE, 2008). Já o termo nocicepção deve ser entendido como os processos neurais de codificação e processamento de estímulos nocivos, é uma definição fisiológica e pode se estabelecer sem que ocorra dor ou vice-versa, mas geralmente estes aparecem associados a patologias dolorosas (LOESER; TREEDE, 2008). Enquanto a dor é um fenômeno subjetivo, a nocicepção é o objeto da fisiologia sensorial, sendo assim, a percepção da dor é complexa e envolve além do componente sensorial, também fatores afetivos e cognitivos (NAVRATILOVA et al., 2010).

A dor pode então ser classificada em diferentes classes, primeiramente a dor associada à proteção do organismo em circunstâncias que podem causar dano potencial aos tecidos é definida como dor nociceptiva (WOOLF, 2010). A dor nociceptiva é gerada pela ativação dos nociceptores que são receptores sensoriais capazes de serem ativados por estímulos nocivos e de transmitir estas informações até estruturas supra-espinhais envolvidas na percepção da dor. Assim, um determinado estímulo nociceptivo, como estímulos mecânicos, térmicos ou ainda químicos, deve ser capaz de ativar os nociceptores expressos na periferia para que cause uma resposta dolorosa no organismo.

Os nociceptores são definidos como terminações periféricas livres de axônios oriundos de neurônios sensoriais nociceptivos, estes neurônios possuem o seu

corpo celular nos gânglios do trigêmio (GT) para a face, nos gânglios da raiz dorsal (GRD) para o corpo, e nos gânglios nodoso (GN) e vagal (GV) para as vísceras (Figura 1). Sendo neurônios pseudo-unipolares os neurônios nociceptivos possuem terminações centrais (medula espinhal e tronco cerebral) e também periféricas (tecido onde possui inervações), e então são capazes de detectar a informação na periferia e transmitir a estruturas centrais. Os neurônios sensoriais envolvidos na transmissão de estímulos nociceptivos podem ser classificados em duas classes principais e apresentam diferenças quanto à velocidade de condução, receptores expressos na membrana e também quanto à categoria de estímulo nocivo conduzido, sendo que as fibras Aδ são formadas por neurônios mielinizados de médio diâmetro, e as fibras C são representadas por neurônios de pequeno diâmetro e não mielinizados (BASBAUM et al., 2009; LOESER; TREEDE, 2008; OSSIPOV; DUSSOR; PORRECA, 2010; WOOLF, 2010).

A transmissão de um determinado estímulo nocivo depende da sua capacidade de gerar um potencial de ação quando da ativação de um nociceptor na periferia, este é então conduzido até o corno dorsal da medula espinhal (sobretudo as lâminas I, II e V), onde então ocorre a liberação de neurotransmissores excitatórios, principalmente substância P (SP) e glutamato. Após a ocorrência deste processo é possível que uma resposta reflexa ao estímulo nocivo seja gerada, e desta forma o local afetado será afastado da fonte de estímulo, esta ação é gerada pela ativação de um neurônio motor reflexo. Porém, para que este sinal seja transmitido até centros supra-espinhais é necessário que um neurônio de segunda ordem sensorial (como neurônios de projeção e interneurônios) seja ativado e então estes sinais nocivos serão prontamente discriminados e reconhecidos como dolorosos (Figura 1). O sinal pode ascender através de axônios provenientes de neurônios de projeção pela via espinotalâmica que apresenta terminações no tálamo ventroposterior e ventrobasal, sendo projetado para o córtex somatosensorial, esta via é importante para o reconhecimento do local e a intensidade do estímulo doloroso. Uma via diferente pode conduzir os estímulos provenientes do corno dorsal da medula espinhal até o córtex insular e cingular, através de conexões no núcleo parabraquial (tronco cerebral) e na amígdala, esta via é denominada espinoparabraquial amidalóide e é relevante para modulação das dimensões afetivas da experiência dolorosa (BASBAUM et al., 2009; LOESER; TREEDE, 2008; OSSIPOV; DUSSOR; PORRECA, 2010; WOOLF, 2010).

Diferente da dor nociceptiva, uma segunda forma de dor, denominada dor inflamatória, é também adaptativa e tem função de proteger o organismo, porém neste caso a sensibilidade sensorial é aumentada após o dano aos tecidos de maneira a auxiliar a recuperação do local lesado. Dessa forma, os pacientes relatam a sensação de dor a estímulos antes descritos como inócuos (dor a estímulos não nociceptivos denominada alodínia), ou o aparecimento de hiperalgesia que é considerada como a percepção exacerbada da dor a estímulos anteriormente descritos como dolorosos, além da ocorrência de dor espontânea (LOESER; TREEDE, 2008; WOOLF, 2010). A dor inflamatória é causada pela ativação do sistema imune após lesão tecidual ou infecção, e esta forma de dor é de fato uma das características principais da inflamação. Mesmo que esta dor seja adaptativa, ainda deve ser reduzida em pacientes com inflamação recorrente, como no caso de pacientes com artrite inflamatória, e em casos de lesão extensa ou grave (CHANDRATRE et al., 2013; LINDSAY et al., 2011; WOOLF, 2010).

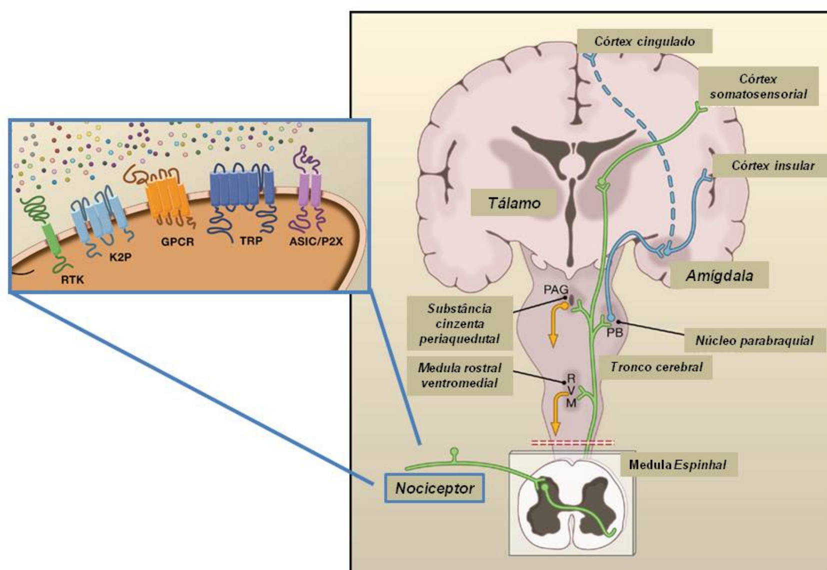

Figura 1 - Anatomia dos mecanismos associados à transmissão de estímulos dolorosos. Adaptado de Basbaum et al. (2009).

Enquanto a dor nociceptiva é um processo agudo que desencadeia respostas apropriadas para a proteção do organismo, e normalmente a dor persiste apenas até a resolução da lesão; a dor crônica ocorre quando a dor tende a continuar após a resolução da lesão ou prossegue devido à modificação dos mecanismos envolvidos na modulação da dor (WOOLF, 2010). Na dor crônica associada à dor inflamatória pode-se observar o aparecimento de hipersensibilidade a estímulos dolorosos e inócuos e também dor espontânea, estes sintomas clínicos são causados pela modificação dos mecanismos usuais da transmissão dos estímulos nociceptivos. Sendo que estes sintomas podem ser vinculados a mecanismos moleculares periféricos ou centrais e estes estão associados ao processo denominado como sensibilização. Neste caso, a ocorrência deste fenômeno pode causar um aumento na resposta de neurônios nociceptivos a estímulos supra-limiares, ou ainda, resposta a estímulos abaixo do limiar de ativação destes. E podem, também, ocorrer descargas espontâneas e aumento do campo receptivo dos nociceptores (BASBAUM et al., 2009; LOESER; TREEDE, 2008; OSSIPOV; DUSSOR; PORRECA, 2010; WOOLF, 2010).

Diversas patologias dolorosas levam a hipersensibilidade e redução da qualidade de vida e dessa forma quando o tratamento não é adequado ou ainda os alvos moleculares conhecidos ainda não são bem esclarecidos, é possível que o tratamento seja problemático (WOOLF, 2010). Dentre estas síndromes dolorosas, temos as artrites inflamatórias, entre elas a artrite úrica (CHANDRATRE et al., 2013; LINDSAY et al., 2011).

1.3 Gota

1.3.1 Histórico

A gota foi uma das primeiras doenças a ser distinguida como uma entidade clínica, sendo primeiramente identificada pelos Egípcios em 2640 a.C., a podagra (ataque de gota agudo ocorrendo na primeira articulação metatarso-falangeana que gera inflamação e dor) foi após reconhecida por Hipócrates no quinto século a.C..

Algumas das percepções clínicas de Hipócrates sobre a gota ainda podem ser consideradas como verdadeiras ainda nos dias de hoje, e esta, era referida como uma doença que reduzia a mobilidade dos pacientes e também estava associada a uma dieta rica em carnes e bebidas alcoólicas, sendo por esse motivo definida como a “artrite dos ricos”. E também mencionada como a “doença dos reis” devido a sua associação a um estilo de vida influente (NUKI; SIMKIN, 2006; SMITH; BRACKEN; SMITH, 2011). Galeno, seis séculos depois, foi o primeiro a descrever os tofos que estavam presentes nas articulações e sob a pele como nódulos duros de pacientes com hiperuricemia prolongada.

A palavra “gota” foi utilizada pela primeira vez para referir-se a podagra pelo monge Dominicano Randolfo de Bocking (1197-1258). Também, Thomas Sydenham, que foi um famoso clínico inglês e possuía artrite úrica, descreveu o ataque agudo de gota como:

> O paciente vai para a cama e dorme serenamente até que cerca de duas horas da manhã ele é despertado por uma dor que geralmente ataca o dedo grande do pé, mas pode também estar presente no calcanhar ou no tornozelo. A dor presenciada parece com aquela de um osso deslocado [...] e esta é rapidamente seguida de um calafrio e ligeira febre [...] a dor [...] que é amena no começo aumenta gradualmente e torna-se mais violenta com o passar das horas... até que se torna tão dolorosa que se torna difícil resistir ao peso das roupas ou até mesmo o balanço do quarto provocado por uma pessoa que anda fortemente nas proximidades (NUKI; SIMKIN, 2006).

Antoni van Leeuwenhoek (1632–1723) um dos pioneiros na microscopia primeiramente identificou e descreveu em 1679 a aparência dos cristais de um tofo de gota como sendo partículas pequenas e transparentes com extremidades pontiagudas, porém a sua constituição química ainda era desconhecida. A identidade química destes tofos foi posteriormente descrita como sendo ácido úrico pelo químico inglês Woolaston (1797), que demonstrou a presença deste em um tofo presente na sua própria orelha. Cerca de 50 anos após (1859), Alfred Baring Garrod propôs que a deposição de urato poderia ser entendida como a causa e não um efeito da inflamação observada na gota (NUKI; SIMKIN, 2006). O suporte experimental para esta hipótese foi obtido posteriormente por uma demonstração feita por Freudweiler, onde demonstrou que a gota aguda poderia ser precipitada pela injeção intra-articular de microcristais de urato monossódico (FREUDWEILER,

1899; NUKI; SIMKIN, 2006), e também foi observado que a formação de tofos poderia ocorrer após a injeção subcutânea de cristais de urato (HIS, 1900; NUKI; SIMKIN, 2006).

Estes experimentos foram particularmente ignorados até que por volta de 1960, quando ocorreu a publicação de um trabalho por McCarty e Hollander, demonstrando que os cristais presentes no fluido sinovial de pacientes com gota era composto por urato monossódico (MCCARTY; HOLLANDER, 1961). O mesmo pesquisador também publicou um trabalho por volta de 1960, onde evidenciou que a presença de cristais de urato monossódico (MSU) estava relacionada à dor e inflamação observada no ataque de gota aguda. Pois os pesquisadores Faires e MacCarty ao administrarem cristais de MSU nas suas próprias articulações presenciaram uma rápida inflamação que apresentava os mesmos sintomas de uma crise aguda de gota, assim os cristais de MSU foram identificados como o sinal inicial para a precipitação do ataque agudo de gota, porém o mecanismo deste ainda não estava esclarecido (FAIRES; MCCARTY, 1962; MARTINON, 2010a).

Mesmo que a gota conte com uma história de mais de 2500 anos e seja considerada uma das doenças mais antigas conhecidas, os mecanismos associados a esta doença ainda estão em estudo, e estes podem ser importantes para a descoberta de novos tratamentos para os sintomas associados a esta artrite dolorosa (MARTINON, 2010a; NUKI; SIMKIN, 2006; SMITH; BRACKEN; SMITH, 2011).

1.3.2. Epidemiologia

A artrite é a causa mais comum de dor e desabilidade física em adultos, levando à redução da qualidade de vida e à procura por cuidados médicos (SINGH, 2009; VANITALLIE, 2010). A gota é caracterizada como uma forma de artrite inflamatória dolorosa, sendo que esta afeta principalmente homens (geralmente após os 40 anos de idade) e também mulheres em período pós-menopausa (principalmente com mais de 60 anos de idade) (RICHETTE; BARDIN, 2010; RODDY; DOHERTY, 2010). No Brasil não existem dados que indiquem a prevalência da artrite úrica na população. Porém já foi descrito que a gota atinge

mais de 1% dos homens adultos no ocidente (MARTINON, 2010a), o que confirma o fato de que esta é artrite inflamatória mais comum entre indivíduos do sexo masculino (RICHETTE; BARDIN, 2010; SAAG; CHOI, 2006). Além disso, a prevalência desta forma de artrite e também de hiperuricemia nos Estados Unidos pode chegar a 4% em indivíduos com mais de 75 anos de idade (NEOGI, 2011).

Diversos fatores podem ser indicativos de uma maior probabilidade para a ocorrência de artrite úrica, e já foi observado que o aparecimento desta doença está associado a fatores modificáveis e não modificáveis (RODDY; DOHERTY, 2010; SAAG; CHOI, 2006). Fatores como: aumento da longevidade, ser do sexo masculino, ou ainda ser do sexo feminino no período pós-menopausa (causa principal é a perda do efeito uricosúrico dos hormônios estrógenos) são considerados como características não modificáveis para uma maior prevalência de gota. Além disso, fatores modificáveis podem também contribuir para um maior risco da incidência de gota, sendo que o principal deles é o nível de urato sérico, porém, a obesidade, determinados medicamentos (aspirina, diuréticos e ciclosporina), dieta contendo grande quantidade de alimentos ricos em purinas (como carnes e frutos do mar) e também bebidas alcoólicas (RODDY; DOHERTY, 2010; SAAG; CHOI, 2006) podem influenciar na presença de gota. A artrite úrica também esta associada a uma série de doenças como a insuficiência renal, síndrome metabólica, hipertensão, e também doenças cardiovasculares (RODDY; DOHERTY, 2010; SAAG; CHOI, 2006).

A prevalência e a incidência da artrite úrica estão aumentando principalmente nos países industrializados, sendo que as principais causas são a maior expectativa de vida da população, e também a uma série de modificações no estilo de vida (como a redução do consumo de alimentos contendo purinas e também de bebidas alcoólicas, e também exercício físico). Dessa maneira, estudos que avaliem os mecanismos envolvidos nos sintomas associados à artrite úrica são de grande importância para melhorias no tratamento dos pacientes (RICHETTE; BARDIN, 2010; RODDY; DOHERTY, 2010; TERKELTAUB, 2010).

1.3.3. Manifestações clínicas da gota

O desenvolvimento patológico da gota pode ser caracterizado por quatro fases: hiperuricemia assintomática, ataques de artrite aguda, períodos intermediários assintomáticos e gota artrítica crônica. Porém a progressão desta doença pode ser diferente entre os pacientes. O primeiro estágio observado na gota é caracterizado por hiperuricemia assintomática, sendo um fator clínico comum e que pode influenciar a predisposição ao desenvolvimento de gota. O risco para o desenvolvimento de gota aumenta com o aumento da hiperuricemia (principalmente maior que 9,0 mg/dL) e com a cronicidade da mesma. Entretanto, apenas um pequeno número de pacientes com hiperuricemia apresentam artrite úrica, e este fenômeno ainda não foi esclarecido. Alguns eventos podem estar associados à hiperuricemia assintomática, como hipertensão e doenças vasculares, indicando que esta condição pode não ser benigna e poderia induzir a estes eventos (MANDELL, 2008; SCHUMACHER, 2008; SMITH; BRACKEN; SMITH, 2011).

Os estágios subsequentes são caracterizados por ataques agudos de gota alternados por períodos assintomáticos. Assim, o aumento da hiperuricemia pode causar a deposição de cristais de MSU nos tecidos articulares, também após trauma ou eventos específicos pode ocorrer a liberação de cristais de MSU no espaço articular e estes podem então causar um processo inflamatório que é caracterizado pelo ataque agudo de gota. Estes ataques são autolimitantes e tendem a se autorresolver em cerca de 7 a 10 dias mesmo na ausência de tratamento (RICHETTE; BARDIN, 2010). Porém, eles tendem a ocorrer novamente, e são acompanhados por períodos intermediários onde mesmo havendo a presença de cristais de MSU nas articulações em níveis menores, não há desenvolvimento de inflamação. Quando da ocorrência do primeiro ataque de gota, a maioria dos pacientes (aproximadamente 60%) desenvolve um segundo ataque agudo em um período de um ano (FERRAZ; O'BRIEN, 1995). Durante o ataque agudo os principais sintomas observados nos pacientes são dor súbita e martirizante (por cerca de 6 a 72 horas), inchaço, vermelhidão, elevação da temperatura e rigidez na articulação, e também febre e calafrios. A artrite úrica aguda afeta geralmente uma única articulação (monoartrite aguda), sendo que na maioria dos casos a principal articulação acometida é a primeira articulação metatarso-falangeana, porém, outras articulações podem ser afetadas como joelho, punho, tarso e tibiotársicas (MANDELL, 2008; SMITH; BRACKEN; SMITH, 2011).

No entanto, se o tratamento para os ataques agudos e para a hiperuricemia não for realizado poderá ocorrer o desenvolvimento de um estágio denominado de gota tofácea crônica (RICHETTE; BARDIN, 2010). Durante esta fase ocorre a formação de tofos nas articulações e tecidos moles, estes são formados por cristais de MSU circundados por células inflamatórias, e podem causar a destruição óssea e deformidade das articulações. A gota tofácea pode se desenvolver em cerca de 5 anos em 30% dos pacientes sem tratamento, estes tofos encontrados principalmente nas extremidades das orelhas e também nas articulações dos dedos das mãos ou pés, são normalmente não dolorosos (SCHUMACHER et al., 2005). Porém, o acúmulo destas estruturas na cavidade articular reduz a capacidade de mobilidade destas articulações e causa a diminuição da qualidade de vida dos pacientes com gota crônica (MANDELL, 2008; RICHETTE; BARDIN, 2010; SMITH; BRACKEN; SMITH, 2011).

1.3.4 Fisiopatologia da inflamação associada à artrite úrica

A síndrome clínica associada à gota é oriunda da deposição de cristais de MSU principalmente nas articulações, onde estes levam a uma resposta inflamatória e dolorosa. O sintoma clássico da gota é denominado podagra e é causado pela deposição de cristais de MSU na articulação do dedo grande do pé (primeira articulação metatarso-falangeana). A deposição de cristais de MSU tende a ocorrer nas articulações quando os níveis séricos de urato se tornam elevados e este é o principal fator que pode desencadear esta forma de artrite inflamatória (RICHETTE; BARDIN, 2010; VANITALLIE, 2010). O ácido úrico é o produto final do metabolismo das purinas nos humanos, sendo produzido pela oxidação destas pela enzima xantina oxidase. Outros mamíferos são capazes de excretar alantoína que é formada a partir do ácido úrico pela ação catalítica da enzima uricase presente nos peroxissomos dos hepatócitos (ROCK; KATAOKA; LAI, 2013). Dessa maneira o ácido úrico é normalmente encontrado como um constituinte usual dos fluidos biológicos ou do meio intracelular em seres humanos. Como o ácido úrico é um ácido fraco (pKa de 5,75) ele se encontra majoritariamente ionizado em pH fisiológico (urato), e diferente da alantoína que apresenta grande solubilidade este

pode precipitar quando em elevadas concentrações. É possível que durante a evolução, os primatas tenham perdido a função da enzima uricase, e dessa maneira quando da ingestão excessiva de alimentos ricos em purinas a elevação dos níveis séricos de urato é um fator relevante para a precipitação de gota (NEOGI, 2011; RICHETTE; BARDIN, 2010; ROCK; KATAOKA; LAI, 2013).

A hiperuricemia é definida como níveis de ácido úrico maiores que 6,8 mg/dL (NEOGI, 2011; TERKELTAUB, 2010). Mesmo que este fator seja comum e seja ainda mais normal em indivíduos com idade avançada, muitos pacientes são assintomáticos mesmo que apresentem hiperuricemia. A hiperuricemia pode ser causada tanto pela produção elevada como pela baixa excreção de ácido úrico. O excesso de urato produzido é excretado principalmente através da urina por ação do rim ou ainda pelo intestino (excreta apenas um terço da produção) (NEOGI, 2011; RICHETTE; BARDIN, 2010). A principal causa que conduz a hiperuricemia em aproximadamente 90% dos pacientes é a hipoexcreção de urato por ação renal. Assim, a excreção pelos rins pode ser reduzida por diversos fatores como insuficiência renal crônica, ingestão de medicamentos que reduzem a excreção de urato, ou ainda alteração na reabsorção de urato. Além disso, o restante dos pacientes com hiperuricemia (cerca de 10%) apresentam uma produção elevada de urato, e assim esta excede a capacidade de filtração pelo glomérulo aumentando então os níveis séricos de urato. A hiperprodução de urato pode ser causada por vários fatores como: deficiência genética (aumento na síntese de ácido úrico por maior atividade das enzimas envolvidas), algumas doenças como neoplasias e psoríase, administração de fármacos (diuréticos, baixas doses de aspirina, quimioterápicos) ou por dieta excessiva contendo alimentos ricos em purinas (NEOGI, 2011; RICHETTE; BARDIN, 2010).

A partir de uma concentração sérica em torno de 6,8 mg/dL de urato é possível que ocorra a precipitação de cristais de urato monossódico, esta é um concentração de saturação para este soluto. Os cristais de urato monossódico são formados pela reação entre o urato que precipita nas articulações e o ânion sódio, sendo que o urato monossódico é mais solúvel que o urato (a 37ºC). Assim, quanto maior a concentração sérica de urato maior a probabilidade que ocorra a deposição deste nas articulações (CHOI; MOUNT; REGINATO, 2005; MARTINON, 2010a). Porém, o risco para que ocorra a precipitação destes cristais nas articulações aumenta na presença de condições que predispõem este fenômenos, entre eles

temos, trauma ou irritação das articulações, temperatura corporal reduzida (extremidades do corpo podem ser mais afetadas e ataques noturnos são mais comuns), alterações no pH (como observado em pacientes com cetose após cirurgia), e também doença prévia de alguma articulação (locais afetados por osteoartrite previamente) (CHOI MOUNT; REGINATO, 2005; NEOGI, 2011; RICHETTE; BARDIN, 2010).

O mecanismo preciso associado à inflamação observada na gota ainda permanece incerto, porém diversos mecanismos moleculares foram postulados e estes parecem contribuir de maneira significativa para a inflamação e dor obervadas no ataque agudo de gota. Quando presente nas articulações os cristais de urato monossódico podem induzir a uma resposta inflamatória que é o fenômeno central envolvido na patologia da gota (RICHETTE; BARDIN, 2010). O sistema imune é capaz de monitorar e detectar moléculas intracelulares que servem como sinais sobre processos de lesão às células e desse modo criam respostas apropriadas quando necessário, auxiliando no reparo do tecido danificado. Diversas moléculas intracelulares são consideradas como sinais de perigo ou ainda descritas como padrões moleculares associados ao dano e o ácido úrico é um desses fatores (na forma de cristais de urato monossódico na gota) (ROCK; KATAOKA; LAI, 2013). A resposta imunológica inata pode ser ativada pelos cristais de MSU, e os principais sensores que já foram identificados para moléculas intracelulares como os cristais de MSU são as proteínas NLR (denominados como receptores do tipo NOD). Quando ativados pelos cristais de MSU as proteínas NLR podem formar complexos citoplasmáticos denominados inflamassomas, e estes são capazes de ativar a caspase-1 (Figura 2). Na gota foi observado que o NALP3 (NLRP3) inflamassoma está associado à dor e inflamação que ocorre após um ataque agudo de gota (MARTINON et al., 2006; SMITH; BRACKEN; SMITH, 2011; VANITALLIE, 2010).

Os cristais de MSU são capazes de ativar uma cascata inflamatória que causa uma resposta de inflamação aguda, mas autolimitante. Dentre um dos mecanismos moleculares descritos até o momento para a gota, está o reconhecimento dos cristais de MSU por receptores de membrana, como receptores do tipo Toll (TLRs) 2 e 4, expressos em fagócitos mononucleares, que podem ser residentes da articulação. Após, estes são capazes de fagocitar os cristais de urato monossódico, este processo também é auxiliado pela proteína adaptadora de TRL, a MyD88, que é capaz de transduzir a ativação ao fator de transcrição nuclear NF-κB,

levando então a expressão de uma variedade de moléculas inflamatórias (como o fator de necrose tumoral, TNF-α; as interleucinas, IL-6 e IL-8; e a cicloxigenase 2, COX-2). Além disso, a fagocitose dos cristais leva a produção e liberação de IL-1β, um processo via ativação do inflamossoma NLRP3 e a ativação posterior da caspase-1 (Figura 2). A ativação do NLRP3 pode ser inibida por concentrações elevadas de colchicina (inibidor de microtúbulo) que é capaz de bloquear a fagocitose dos cristais de MSU. Assim, a precipitação dos cristais de MSU leva a ativação de diversas células, como sinoviócitos, macrófagos e leucócitos de maneira dependente ou não da produção de IL-1β, e estes tem a capacidade de produzir uma grande variedade de mediadores inflamatórios (como por exemplo, as quimiocinas CXCL1 e 8) (MARTINON, 2010a; 2010b; RICHETTE; BARDIN, 2010; SMITH; BRACKEN; SMITH, 2011; TERKELTAUB, 2009).

Dessa maneira, após a deposição dos cristais de MSU, estes são capazes de induzir a migração de neutrófilos para as articulações, por mecanismos dependentes da ação da IL-1 e TNF-α, que podem estimular o endotélio vascular a expressar moléculas de adesão para leucócitos. A ativação das células endoteliais dos vasos conduz a um aumento a permeabilidade vascular causada principalmente pelo aumento do fluxo sanguíneo e também da vasodilatação, estes fenômenos estão associados a um aumento da expressão de moléculas de adesão para leucócitos. Todos estes fatores auxiliam na migração dos leucócitos para a articulação onde estes podem amplificar a resposta, pela ativação posterior dos mesmos pelos cristais de MSU com a liberação de citocinas inflamatórias (MARTINON, 2010a; 2010b; RICHETTE; BARDIN, 2010; SMITH; BRACKEN; SMITH, 2011). Além destas células que podem infiltrar na articulação, macrófagos residentes na articulação (sinoviócitos do tipo A) também podem atuar na produção de mediadores inflamatórios (MALAWISTA; DE BOISFLEURY; NACCACHE, 2011; SCHUMACHER; PHELPS; AGUDELO, 1974).

O acúmulo de neutrófilos é então observado na membrana sinovial e também no líquido sinovial, sendo um dos principais eventos associados à sintomatologia do ataque agudo de gota. Além disso, quando da infiltração dos neutrófilos na articulação estes podem também contribuir para a produção de espécies reativas de oxigênio (ROS; como o íon superóxido e o peróxido de hidrogênio - H_2O_2) (MARTINON, 2010a, 2010b; RICHETTE; BARDIN, 2010; SMITH; BRACKEN; SMITH; 2011). De tal modo, estudos têm demonstrado que a fagocitose dos cristais

de MSU por neutrófilos ou células residentes da articulação aumentaria a produção de substâncias oxidantes e que este fenômeno poderia ser inibido pela adição de colchicina (CHIA; GRAINGER; HARPER, 2008; GAUDRY et al., 1993; MARTIN et al., 2010; MARTIN; WALTON; HARPER, 2009). Do mesmo modo, foi observado que pacientes com gota apresentam um nível aumentado de espécies oxidantes em comparação com indivíduos saudáveis, um fator que poderia contribuir para a fisiopatologia desta doença (AMARAL et al., 2012; TERKELTAUB, 2010). Além disso, já foi demonstrado que a vitamina C, um poderoso antioxidante, poderia reduzir a incidência de gota (CHEN et al., 2011; HUANG et al., 2005; JURASCHEK et al., 2011; SHEN; JI, 2011).

Mesmo que os principais indícios demonstrem que a dor observada nos ataques agudos de gota parece ser causada por respostas desencadeadas após a ativação de neutrófilos pelos cristais de MSU, já foi observado que ataques agudos de gota podem ocorrer sem a migração de neutrófilos para a articulação (HOROWITZ et al., 1990). Dessa maneira, ainda não está esclarecido como a dor pode ser processada antes que ocorra a migração de neutrófilos na articulação e se mecanismos moleculares podem ser ativados previamente a estes fenômenos.

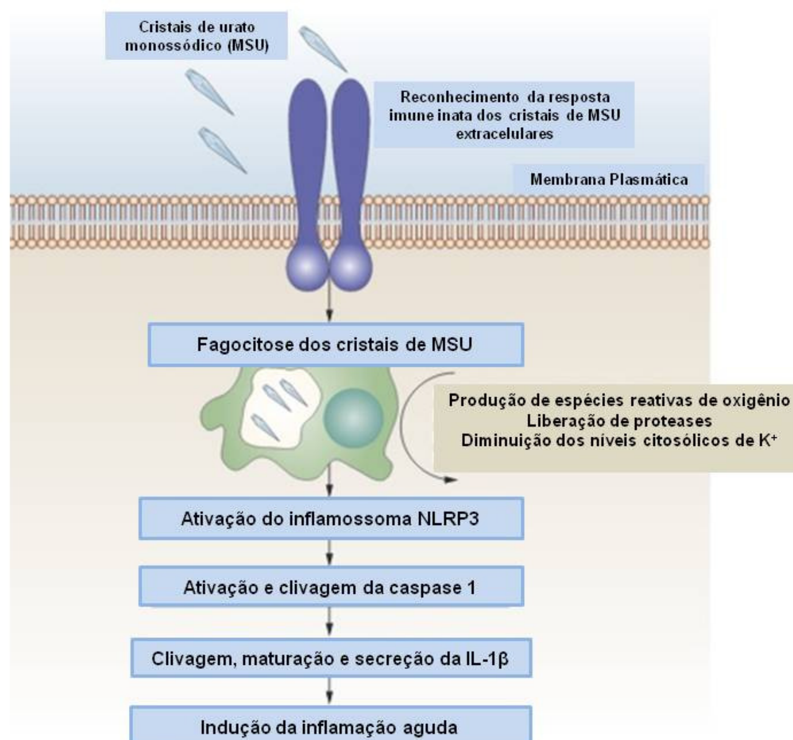

Figura 2 - Mecanismos associados à inflamação aguda observada na gota. Adaptado de Terkeltaub (2010).

1.3.5 Diagnóstico e tratamento da gota

A Liga Europeia Contra o Reumatismo (EULAR) propôs algumas recomendações chaves para o diagnóstico apropriado da gota (SIVERA et al., 2013). Primeiramente é descrito que o desenvolvimento em ataques agudos de dor grave, inchaço, e vermelhidão nas articulações podem ser sugestivos de inflamação provocada por cristais, porém não é específico para gota. Mesmo que as apresentações típicas de gota como hiperuricemia e podagra possam ocorrer em períodos recorrentes o diagnóstico clínico de gota não é definitivo, pois para que o diagnóstico de gota seja adotado na clínica é necessário fundamentalmente que os cristais de MSU sejam identificados na articulação. Este é considerando então o padrão necessário para a determinação do diagnóstico, e este pode ser feito através

de amostras obtidas por aspiração de tofos ou por amostras de fluídos sinoviais. Mesmo que o principal risco para a gota seja a hiperuricemia elevada e crônica, este sintoma não confirma a presença de gota, pois muitas pessoas que apresentam hiperuricemia não desenvolvem gota, e durante os ataques agudos os níveis séricos de urato podem não estar elevados em pacientes com gota (SIVERA et al., 2013; SMITH; BRACKEN; SMITH, 2011).

Além disso, a excreção renal de ácido úrico deve ser avaliada em pacientes selecionados principalmente aqueles com histórico familiar de gota de aparecimento prematuro (pacientes com menos de 25 anos), ou que possuam cálculos renais. E também fatores de risco para a gota devem ser observados, incluindo características de síndrome metabólica, como obesidade, hiperglicemia, hiperlipidemia e hipertensão. Técnicas emergentes têm sido utilizadas para o monitoramento da progressão da doença e também para avaliar a resposta ao tratamento, dentre elas temos a ultrassonografia, a tomografia computadorizada com energia dual, e também tomografia de ressonância nuclear magnética (MRI, utilizada particularmente para detecção do envolvimento espinhal na gota) (SIVERA et al., 2013; SMITH; BRACKEN; SMITH, 2011; VANITALLIE, 2010).

A terapia utilizada para o tratamento da gota pode ser geralmente dividida no tratamento para os ataques agudos e estratégias para profilaxia e redução dos episódios de crises agudas (SMITH; BRACKEN; SMITH, 2011; SURESH; DAS, 2012; TERKELTAUB, 2010). Primeiramente, a terapia para reduzir a resposta inflamatória e a dor encontrada nos ataques agudos é realizada principalmente com o uso de colchicina, anti-inflamatórios não esteroidais (AINES) e glicocorticóides. O uso de AINES e também da colchicina por via sistêmica são normalmente os mais indicados, e o tratamento deve ser iniciado o mais rápido possível (tratamento abortivo) para que estes possam ser mais efetivos no tratamento. Porém, a escolha do agente terapêutico mais apropriado, dose e duração da terapia devem ser decididas levando em conta as características do paciente e comorbidades associadas que limitam o uso de agentes específicos, a gravidade da gota e também as circunstâncias clínicas observadas. Como a colchicina (*Colchicum autumnale*) é pouco tolerada as doses administradas devem ser mínimas, os efeitos tóxicos da colchicina tendem a serem maiores em pacientes idosos e aqueles com falência hepática ou renal, ou ainda quando administrada conjuntamente com fármacos específicos (ciclosporina, verapamil e drogas para redução de lipídeos). Além disso,

os AINES não são bem tolerados em pacientes pela indução de lesões gastroduodenais e também não é indicado no tratamento para pacientes com gastrites, úlcera péptica, insuficiência renal, problemas hepáticos e hipertensão (RICHETTE; BARDIN, 2010; SMITH; BRACKEN; SMITH, 2011; SURESH; DAS, 2012; TERKELTAUB, 2009, 2010).

A administração de corticóides se mostra efetiva e pode ser utilizada por administração por via intra-articular quando principalmente apenas uma articulação estiver envolvida. Também a administração de glicocorticóides pode ser realizada por administração oral ou ainda intramuscular, porém o tratamento com corticóides deve ser breve devido aos efeitos adversos associados. Além disso, medidas comuns devem ser tomadas durante um ataque agudo de gota como: alterar a dieta, descanso, descontinuar a ingestão de álcool e de outros agentes precipitantes de crises agudas (uso de diurético, por exemplo). Novas terapias para os ataques agudos estão em estudo como os antagonistas (anakinra, um antagonista do receptor para IL-1β recombinante humano) e os inibidores (canakinumab, anticorpo para IL-1β humana) da IL-1β, porém maiores evidências clínicas devem ser obtidas para avaliar a eficácia destes agentes para o tratamento da gota aguda. Além disso, o possível desenvolvimento de infecções com o uso de inibidores de citocinas é um dos principais efeitos adversos observados (RICHETTE; BARDIN, 2010; SMITH; BRACKEN; SMITH, 2011; SURESH; DAS, 2012; TERKELTAUB, 2009, 2010).

A terapia crônica para a gota tem como objetivos prevenir os ataques agudos e também reduzir as concentrações de urato, a recomendação usual é que a terapia inicie quando o paciente apresentar dois ataques agudos de gota em um ano. Como cerca de 60% dos pacientes normalmente são atingidos com um segundo ataque dentro de um período de um ano após o primeiro ataque, então a maioria dos pacientes que apresentam um primeiro ataque devem iniciar a terapia para a redução de urato. O controle prolongado da hiperuricemia em pacientes com gota consiste na terapia com inibidores da xantina oxidase (alopurinol e febuxostat), uricosúricos como a probenecida e a benzbromarona, e também agentes com atividade de uricase. Com o uso destes agentes a concentração de urato deve ser mantida abaixo do ponto de saturação, e é possível que também ocorra um auxílio na dissolução dos depósitos de cristais. Porém a decisão para o uso destas terapias deve ser medida pelo aparecimento de efeitos adversos, e também mudanças no estilo de vida dos pacientes devem ser recomendadas (RICHETTE; BARDIN, 2010;

SMITH; BRACKEN; SMITH, 2011; SURESH; DAS, 2012; TERKELTAUB, 2009, 2010).

O fármaco mais utilizado é o alopurinol, um inibidor da atividade da xantina oxidase, mas este agente pode causar síndrome de hipersensibilidade (com sintomas associados de febre, problemas hepáticos, falência renal e também intolerância gastrointestinal), e seu uso deve ser restringido em pacientes com insuficiência renal e idosos. O uso de outro inibidor da xantina oxidase (febuxostat) é útil em pacientes com intolerância ao alopurinol, sendo que este fármaco também pode induzir a efeitos adversos como aumento da atividade de enzimas hepáticas e um pequeno aumento na taxa de eventos cardiovasculares sérios, e assim o seu uso em pacientes isquêmicos ou com falência cardíaca congestiva deve ser evitado. Agentes uricosúricos podem ser utilizados como segunda linha de tratamento para pacientes com baixa excreção de ácido úrico, assim estes fármacos como a probenecida e a benzbromarona devem ser usados como alternativas ao uso de alopurinol em pacientes com função renal normal, mas são contraindicados em pacientes com urolitíase (RICHETTE; BARDIN, 2010; SMITH; BRACKEN; SMITH, 2011; SURESH; DAS, 2012; TERKELTAUB, 2009, 2010).

O uso de agentes que possam funcionar como a uricase é uma nova alternativa no tratamento agudo da gota, estes fármacos inovadores são capazes de reduzir a concentração de urato em pacientes refratários aos tratamentos convencionais. A pegloticase catalisa a oxidação do ácido úrico solúvel em alantoína, como a expressão da uricase é ausente em humanos, este processo auxilia na excreção do ácido úrico. Este fármaco foi aprovado pelo Food and Drug Administration (FDA) nos Estados Unidos para o tratamento da gota crônica em pacientes adultos refratários as terapias convencionais, e é uma forma recombinante da uricase conjugada com polietilenoglicol, de maneira a suprimir a imunogenicidade à esta substância e aumentar a meia-vida da mesma. Porém, reações adversas associadas ao desenvolvimento de anticorpos contra este fármaco (ataques anafiláticos), reações indesejáveis à via de administração intravenosa (eritema, prurido e dor no peito), ou ainda desenvolvimento de artralgia, náuseas, anemia foram descritos após a administração de pegloticase. Assim, o uso deste fármaco ainda deve ser avaliado observando os risco e benefícios para pacientes com gota crônica (SCHLESINGER; YASOTHAN; KIRKPATRICK, 2011; SMITH; BRACKEN; SMITH, 2011; TERKELTAUB, 2010).

Finalmente é possível observar que a terapia para a artrite úrica encontra algumas limitações principalmente relacionadas aos efeitos colaterais das drogas disponíveis e a redução parcial (em geral somente 50%) da dor, sendo que esta ocorre geralmente após 2-3 dias de tratamento (TERKELTAUB, 2009, 2010). Assim, tem se dado ênfase ao desenvolvimento de novos fármacos e também a procura por alvos terapêuticos envolvidos no desenvolvimento da dor e inflamação observadas na gota (BURNS; WORTMANN, 2011; RICHETTE; BARDIN, 2010; TERKELTAUB, 2010).

1.3.6 Dor relacionada à gota e impacto na qualidade de vida

A dor observada em pacientes com gota aguda e crônica parece reduzir de forma significativa a qualidade de vida das pacientes, devido principalmente a impor limitações às atividades usuais e também levar a desabilidade (SINGH, 2009). Além disso, pacientes com artrite úrica mostram uma redução na sociabilidade maior que aqueles pacientes com hipertensão, angina ou diabetes (BRIXNER; HO, 2005). A redução da capacidade de trabalho e a perda de dias de trabalho também podem ser correlacionadas com os ataques agudos de gota, e estes ataques em pacientes refratários às terapias convencionais significativamente afetam a produtividade no trabalho e atividades sociais (EDWARDS et al., 2011). Já foi observado que alguns tratamentos induzem mais rapidamente a um aumento na qualidade de vida de pacientes com artrite úrica em relação à outras terapias (KHANNA et al., 2011; SMITH; BRACKEN; SMITH, 2011), e então podemos observar que o tratamento eficaz da gota pode modificar parâmetros importantes relacionados a percepção dolorosa deste tipo de artrite.

A dor relacionada aos ataques agudos de gota é descrita pelos pacientes como uma dor excruciante que inicia de forma súbita (LINDSAY et al., 2011). Acredita-se que a dor e a inflamação relacionadas à gota sejam induzidas por mecanismos subsequentes à fagocitose e a ativação de neutrófilos por cristais de urato monossódico. Estes, uma vez ativados, são capazes de liberar vários mediadores inflamatórios (BURNS; WORTMANN, 2011; CHOI; MOUNT; REGINATO, 2005; MARTINON, 2010a). Porém, ataques agudos de gota podem

ocorrer mesmo na ausência de infiltração de leucócitos na articulação (HOROWITZ et al., 1990). Além disto, quando ocorre infiltração leucocitária a dor inicia-se antes da detecção de neutrófilos na articulação (HOFFMEISTER et al., 2011; SCHUMACHER; PHELPS; AGUDELO, 1974). Desta forma, células residentes no tecido articular, como sinoviócitos, macrófagos, e os próprios neurônios sensoriais, poderiam realizar o reconhecimento inicial dos cristais de MSU. Porém ainda é pouco esclarecido sobre os mecanismos celulares e moleculares envolvidos na fase inicial da indução de dor e inflamação no ataque agudo de gota.

As fibras sensoriais expressam diversos tipos de canais iônicos essenciais para a detecção e transmissão de estímulos dolorosos, dentre eles o receptor TRPA1 (receptor de potencial transitório anquirina 1) (MORAN et al., 2011). O receptor TRPA1 foi originalmente proposto como um sensor ao frio nocivo (STORY et al., 2003). Atualmente é um alvo de particular interesse devido a sua expressão em neurônios nociceptivos e sua capacidade de transdução de uma grande variedade de estímulos nocivos (CVETKOV et al., 2011; MORAN et al., 2011). Este canal é um dos principais sensores para diversas substâncias oxidantes endógenas (como o H_2O_2) (ANDERSSON et al., 2008; ANDRE et al., 2008; BARALDI et al., 2010; BESSAC et al., 2008; KEEBLE et al., 2009; SAWADA et al., 2008).

Curiosamente o H_2O_2 está implicado na dor causada pela ativação de neurônios sensoriais, como as dores relacionadas à isquemia cardíaca, à inflamação cutânea e à neuropatia induzida pelo diabetes (HILL; SCHAEFER, 2009; NASSINI et al., 2011; POP-BUSUI; SIMA; STEVENS, 2006; SCHULTZ; USTINOVA, 1998). Porém, ainda não foi observado se o H_2O_2 poderia estar envolvido na indução da dor em artrites, como a gotosa, e qual o papel do receptor TRPA1 neste processo. É interessante observar que um subtipo de sinoviócitos (tipo A ou semelhantes a macrófagos) pode fagocitar cristais de MSU (SCHUMACHER; PHELPS; AGUDELO, 1974). Como o processo de fagocitose por leucócitos pode normalmente desencadear a produção de H_2O_2 (SIMCHOWITZ; ATKINSON; SPILBERG, 1982; STANOJEVIC et al., 2008) e este pode estimular o receptor TRPA1 em neurônios sensoriais, isto poderia ser um importante mecanismo responsável pela indução de dor na fase inicial do ataque agudo de gota.

De fato, alguns dados sugerem a possível relação do receptor TRPA1 na artrite gotosa. Primeiramente, sabe-se que uma das características clássicas da descrição da dor relacionada à gota é “como se tivesse sido jogada água fria sobre

as articulações" (NUKI; SIMKIN, 2006; VANITALLIE, 2010). Curiosamente é relatado que o desenvolvimento de hipersensibilidade ao frio em diversos modelos experimentais de dor é dependente do receptor TRPA1 (BARALDI et al., 2010; MORAN et al., 2011). Também, estudos prévios do nosso grupo de pesquisa demonstraram que o canal TRPV1 (TRP vanilóide 1) parece mediar a resposta nociceptiva e edematogênica induzida pela injeção de cristais de MSU em ratos (HOFFMEISTER et al., 2011). Também é importante citar que o receptor TRPA1 pode ser ativado pelo influxo de cálcio mediado pelo receptor TRPV1 (PATIL; JESKE; AKOPIAN, 2010). Além disso, a capsaicina (o principio ativo da pimenta vermelha e agonista do receptor TRPV1) pode ser utilizada como um tratamento tópico para a redução da dor em pacientes com algumas formas de artrite (DEAL et al., 1991) e pode reduzir a dor observada na gota (WALKER; MCCLEANE, 2002). Ainda, os agonistas TRPV1, como a capsaicina e a resiniferatoxina, são capazes de apresentar um efeito analgésico por provocarem degeneração de fibras sensoriais específicas contendo TRPV1 e também TRPA1 (ANAND; BLEY, 2011; DERRY et al., 2013; PECZE et al., 2009; SCHUMACHER, 2010).

Assim especula-se que o receptor TRPA1 poderia ter um papel importante na dor e na inflamação observadas em pacientes com gota. Dessa maneira, a presente pesquisa poderá auxiliar para futuras descobertas de alternativas para o tratamento da dor e inflamação observadas na gota.

1.4 Receptor TRPA1

1.4.1 Receptores de potencial transitório – TRP

Os receptores de potencial transitório são sensores celulares polimodais envolvidos em uma grande variedade de processos celulares (MONTELL, 2005; MORAN et al., 2011). Atualmente, mais de 50 membros da família TRP já foram caracterizados, sendo que a família TRP é das maiores famílias de canais iônicos e apresenta uma ampla distribuição filogenética (MINKE, 2010; VRIENS; APPENDINO; NILIUS, 2009). Em mamíferos a família de canais TRP consiste de 28

diferentes proteínas agrupadas em 6 famílias de acordo com a sua sequência de aminoácidos e sendo designadas como: TRPV (vanilóide), TRPC (canônico), TRPM (melastatina), TRPP (policistina), TRPML (mucolipina) e TRPA (anquirina) (CLAPHAM, 2003; MONTELL; BIRNBAUMER; FLOCKERZI, 2002).

Os canais TRP consistem estruturalmente de seis domínios transmembrana (S1-S6) sendo que ambas as regiões amino- (NH_2) e carboxi- (COOH) terminais estão localizadas intracelularmente possuindo comprimentos variáveis. A porção carboxi-terminal é altamente conservada nos diferentes receptores TRP, e pode conter uma sequência conservada composta de 23 a 25 resíduos de aminoácidos (domínio TRP), presente nas famílias TRPC, TRPN e TRPM e é utilizada como uma região para marcação e descoberta de novos receptores desta família (VENKATACHALAM; MONTELL, 2007; ZHU, 2005). Porém a região amino-terminal normalmente pode conter diversos repetições de anquirina, que medeiam interações específicas entre proteínas e os receptores TRP, e podem estar envolvidas na formação de complexos macromoleculares entre a membrana plasmática e o citoesqueleto (MONTELL, 2005; SEDGWICK; SMERDON, 1999; VENKATACHALAM; MONTELL, 2007).

Mutações nos receptores TRP têm sido relacionadas ao desenvolvimento de diferentes doenças, mostrando a importância destes canais em processos fisiológicos (LEVINE; ALESSANDRI-HABER, 2007; NILIUS, 2007). Recentemente foi descrita que uma mutação com ganho de função para o receptor TRPA1 poderia levar a uma síndrome familiar de dor episódica, demonstrando a importância deste receptor para a transdução de estímulos dolorosos (KREMEYER et al., 2010).

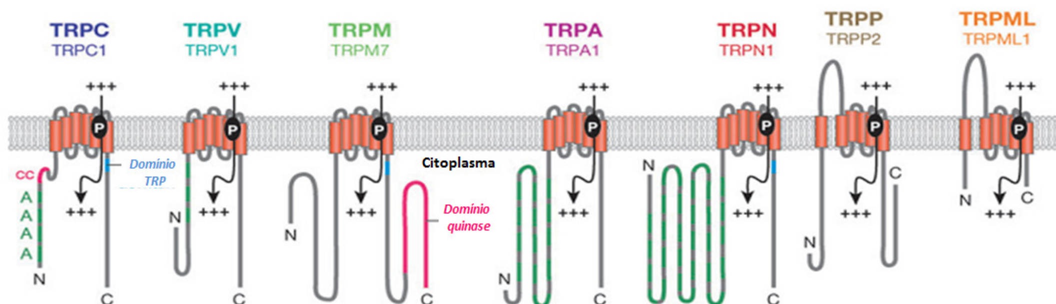

Figura 3 - Representação espacial da estrutura das diferentes famílias de receptores TRP. Adaptado de Montell (2005).

Diversos receptores expressos em neurônios sensoriais são importantes para a detecção de estímulos nocivos, como os receptores TRPV1 e TRPA1. Então, estes canais têm sido indicados como importantes alvos para a descoberta de novos analgésicos, e grande atenção tem sido direcionada a estudos do papel destes receptores em patologias dolorosas. Plantas e extratos contendo ativadores do receptor TRPV1, como a pimenta vermelha (*Capsicum* sp.) são utilizados na culinária e para uso medicinal a milhares de anos (CALIXTO et al., 2005; SCHUMACHER, 2010). Porém, a base molecular do receptor vanilóide foi apenas elucidada a partir do isolamento do clone de DNA complementar (DNAc) que estava relacionado com o receptor ativado pela capsaicina em 1997 (CATERINA et al., 1997).

O receptor TRPV1 foi o primeiro a ser descoberto dos receptores da família vanilóide (TRPV1-TRPV6) (WU; SWEET; CLAPHAM, 2010), é um canal iônico não seletivo para cátions, porém com preferência à cálcio, encontrado nos gânglios do trigêmio e gânglios da raiz dorsal. Este canal é também ativado pela resiniferatoxina (isolada da planta *Euphorbia resinífera*), por calor nocivo (>43ºC) e pH abaixo do fisiológico (CATERINA et al., 1997; TOMINAGA et al., 1998). Uma propriedade única entre os receptores TRPV1 e TRPA1 é que estes estão co-localizados em uma subpopulação de fibras sensoriais peptidérgicas não mielinizadas ou pouco mielinizadas, fibras C ou Aδ. Assim a ativação destes receptores provoca a liberação de neuropeptídeos, como a substancia P (SP) e o peptídeo relacionado ao gene da calcitonina (CGRP).

Estudos iniciais com a aplicação de capsaicina na pele de humanos mostraram que esta era capaz de induzir dor em queimação e vasodilatação, porém após este estado doloroso era seguido por um período refratário, onde ocorria a dessensibilização ao estímulo. Assim, a região previamente exposta era resistente à aplicação posterior de capsaicina ou até mesmo outros estímulos dolorosos como calor nocivo ou substâncias químicas irritantes (CALIXTO et al., 2005; SCHUMACHER, 2010). Além disso, o tratamento com capsaicina em animais adultos ou neonatos pode provocar a degeneração de fibras sensoriais específicas (fibras principalmente de pequeno diâmetro e peptidérgicas) produzindo um efeito analgésico (CALIXTO et al., 2005; SCHUMACHER, 2010), um fenômeno similar é induzido pela administração de RTX (PECZE et al., 2009). Este fenômeno tem sido

explorado para a indução de analgesia em diversas patologias dolorosas (ANAND; BLEY, 2011; DERRY et al., 2013; SCHUMACHER, 2010). Devido à co-localização entre os receptores TRPV1 e TRPA1, é descrito que a dessensibilização utilizando agonistas TRPV1 poderia reduzir também as respostas à agonistas TRPA1 (PECZE et al., 2009).

O receptor TRPV1 devido a sua capacidade de integrar uma série de estímulos químicos e físicos que promovem dor foi considerado com um alvo em potencial para o desenvolvimento de analgésicos (SZALLASI et al., 2007). Dessa maneira, o bloqueio deste receptor utilizando antagonistas específicos ou ainda o uso de agonistas foram as principais estratégias utilizadas para promover o tratamento de diversas síndromes dolorosas (LEVINE; ALESSANDRI-HABER, 2007; PATAPOUTIAN; TATE; WOOLF, 2009; WONG; GAVVA, 2009). Porém, mesmo que diversas evidências experimentais tenham demonstrado a eficácia dos antagonistas TRPV1 em modelos de dor inflamatória ou neuropática, o desenvolvimento de grave hipertermia após administração destes compostos em animais e humanos provocou uma redução no interesse por esta classe de antagonistas (GAVVA et al., 2008; MORAN et al., 2011).

1.4.2 Receptor de potencial transitório anquirina 1 – TRPA1

1.4.2.1 Aspectos gerais do receptor TRPA1

O receptor TRPA1 foi isolado primeiramente em culturas de fibroblastos de pulmão fetal humano (JAQUEMAR; SCHENKER; TRUEB, 1999), e este canal é o único membro da subfamília TRPA identificado em mamíferos (ANDRADE; MEOTTI; CALIXTO, 2012). Esse é descrito como um canal catiônico não seletivo, formado por seis domínios transmembrana contendo suas porções amino- e carboxi-terminais localizadas intracelularmente. Este canal possui aproximadamente 14 repetições do tipo anquirina em seu longo domínio amino-terminal (CVETKOV et al., 2011), e também diversos resíduos de cisteína que conferem a capacidade deste receptor de ser ativado por compostos reativos através de modificação destes resíduos

(ANDRADE; MEOTTI; CALIXTO, 2012; BARALDI et al., 2010). O receptor TRPA1 é geralmente expresso em sub-tipos específicos de neurônios sensoriais peptidérgicos nos gânglios da raiz dorsal, nos gânglios trigeminais, nos gânglios nodoso e vagal (NAGATA et al., 2005; STORY et al., 2003). Porém, este receptor é também expresso em outras células e tecidos, incluindo coração, queratinócitos, pâncreas e cérebro (ATOYAN; SHANDER; BOTCHKAREVA, 2009), bexiga urinária (STRENG et al., 2008), sinoviócitos (KOCHUKOV et al., 2006), entre outros (ANDRADE; MEOTTI; CALIXTO, 2012).

Este canal é um dos principais sensores para compostos isolados de produtos naturais de ação irritante (Figura 4), como o cinamaldeído (encontrado na canela), o isotiocianato de alila (AITC, presente no óleo de mostarda) (JORDT et al., 2004) e a alicina (encontrada no alho) (BAUTISTA et al., 2005). Além disso, o TRPA1 é também estimulado por diversos poluentes industriais e irritantes ambientais (substâncias oxidantes exógenas), como a acroleína (2-propenal, encontrada na fumaça de cigarro), o hipoclorito (–OCl), o dióxido de carbono, e a formalina (utilizada para induzir nocicepção em animais de laboratório) (ANDRADE MEOTTI; CALIXTO, 2012; BARALDI et al., 2010; MCNAMARA et al., 2007). Este receptor é também ativado por compostos endógenos reativos produzidos após dano tecidual e associados a condições dolorosas (Figura 4), como o 4-hidroxinonenal (4-HNE), prostanglandinas, e espécies reativas de oxigênio, como o H_2O_2, ou de nitrogênio, como o peróxido nitrito (ONOO) (ANDERSSON et al., 2008; ANDRE et al., 2008; BARALDI et al., 2010; BESSAC et al., 2008; KEEBLE et al., 2009; SAWADA et al., 2008).

Então a interação entre o receptor TRPA1 e estes compostos altamente reativos é de grande relevância para o reconhecimento do dano celular, principalmente durante eventos inflamatórios, assim este canal pode ser entendido como um sensor para esta modalidade de estímulo (Figura 4). Mesmo, que o receptor TRPA1 seja capaz de ser ativado por uma miríade de compostos oxidantes estruturalmente diversos, estas substâncias apresentam alguns mecanismos de ativação em comum. A maioria dos agonistas TRPA1 são compostos eletrófilos reativos e então são capazes de formar ligação covalente com grupos nucleofílicos, como resíduos de cisteína presentes na região amino-terminal citoplasmática do canal (BANG; HWANG, 2009; HINMAN et al., 2006; MACPHERSON et al., 2007). A modificação covalente dos resíduos de cisteína ocorre por adição de Michael

causada pelos agonistas, sendo que tanto o cinamaldeído como o AITC são capazes de provocar a formação de um ditiocarbamato com o receptor. Uma possibilidade diferente é aquela induzida pelo H_2O_2, causando a formação de uma ligação dissulfeto no receptor por oxidação (ANDERSSON et al., 2008; BANG; HWANG, 2009; CEBI; KOERT, 2007; HINMAN et al., 2006; MACPHERSON et al., 2007). A modificação eletrofílica os resíduos de cisteína na porção amino-terminal do canal TRPA1 provoca a dilatação e permeação principalmente de íons cálcio através do poro.

O receptor TRPA1 pode ser encontrado tanto na periferia, como nos terminações centrais da medula espinhal, dessa maneira a ativação deste canal induz influxo de cálcio causando a liberação de CGRP e SP e também pode contribuir para a geração de potenciais de ação (BASBAUM et al., 2009). Nas terminações centrais, a liberação deste neuropeptídeos conduz a transmissão dos estímulos nociceptivos a estruturas centrais, enquanto que na periferia a estimulação dos nociceptores contendo TRPA1 gera uma série de respostas pro-inflamatórias, um fenômeno denominado de inflamação neurogênica (GEPPETTI et al., 2008; RICHARDSON; VASKO, 2002). As respostas associadas à inflamação neurogênica compreendem fatores vasculares (vasodilatação arteriolar e edema causado por extravasamento plasmático das vênulas pós-capilares) e não vasculares (bronconstrição, secreção glandular de muco, entre outras respostas). Dessa maneira, a ativação do receptor TRPA1 é importante para a patofisiologia de diversas doenças associadas à inflamação neurogênica, como a dor inflamatória, a enxaqueca, a asma e a dermatite (ANDRADE; MEOTTI; CALIXTO, 2012; BAUTISTA; PELLEGRINO; TSUNOZAKI, 2013; NILIUS; APPENDINO; OWSIANIK, 2012).

Outra relevante característica do receptor TRPA1 é que ele é capaz de cooperar com o receptor vanilóide TRPV1, e já foi demonstrado que algumas características funcionais do canal TRPA1 dependem da sua habilidade de interagir com o TRPV1 em neurônios sensoriais (SALAS; HARGREAVES; AKOPIAN, 2009). Sendo que, em condições inflamatórias a relação entre estes canais é importante para a ação estimulatória de mediadores endógenos como a bradicinina e o cálcio (BAUTISTA et al., 2006; PATIL; JESKE; AKOPIAN, 2010). Além disso, o influxo de íons cálcio através do receptor TRPV1 é importante para a ativação posterior do receptor TRPA1 (PATIL; JESKE; AKOPIAN, 2010).

Este canal também pode ser sensibilizado por mediadores endógenos através de vias de segundos mensageiros que pode causar a modificação da atividade do receptor (fosforilação). Estes eventos auxiliam para o aumento da resposta e uma diminuição do limiar de ativação das fibras nociceptivas periféricas em um processo denominado de sensibilização periférica (LOESER; TREEDE, 2008; SZALLASI et al., 2007). O receptor TRPA1 pode ser sensibilizado por uma série de estímulos, e como já foi demonstrado para receptores TRP estes podem ter as suas respostas alteradas por fatores associados à ativação de receptores acoplados à proteína G (GPCR) (CLAPHAM, 2003). Assim, a bradicinina, um mediador não peptídico produzido durante dano tecidual, pode alterar a ativação do canal TRPA1 por interagir com o seu receptor B2 (GPCR) (CALIXTO et al., 2004; BAUTISTA et al., 2006), e causar a ativação da fosfolipase C (PLC) (BANDELL et al., 2004). A estimulação da PLC leva posteriormente a produção de diacilglicerol (DAG) e inositol-1,4,5-trifosfato (IP3), a partir do fosfoinosídeo, fosfatidil-inositol-4,5-bifosfato (PIP2). Consequentemente, estes segundo mensageiros são capazes de induzir a liberação de cálcio do retículo endoplasmático, mediado pelo IP3, ou ainda causar a ativação da proteína cinase C (PKC) induzida por DAG. Porém, ainda não são bem esclarecidos os mecanismos que levam a sensibilização do receptor TRPA1 após ativação de receptor GPCR, porém a ativação da PKC parece não estar envolvido neste processo (DAI et al., 2007; WANG et al., 2008). Também, a estimulação da cinase dependente de AMP cíclico (proteína cinase A, PKA) por mecanismos induzidos pela bradicinina podem sensibilizar o receptor TRPA1 (WANG et al., 2008). Tanto a PKA, como a PKC, são capazes de fosforilar o receptor TRPV1 induzindo um aumento da sua atividade (sensibilização) o que poderia aumentar o influxo de cálcio e provocar sensibilização posterior do receptor TRPA1 (BAUTISTA et al., 2006; DAI et al., 2007; WANG et al., 2008). O receptor TRPA1 ainda pode ser sensibilizado por mecanismos similares aos descritos para a bradicinina por ativação da PLC, quando da estimulação dos receptores ativados por protease, as proteases são mediadores formados em processos inflamatórios (DAI et al., 2007). O receptor TRPA1 então além de ser um sensor para moléculas provenientes do dano celular também pode ser sensibilizado em situações de lesão celular, contribuindo para a amplificação destas respostas.

1.4.2.2 Participação do receptor TRPA1 em patologias dolorosas

De maneira interessante o receptor TRPA1 está também envolvido no desenvolvimento de hipersensibilidade a estímulos mecânicos e ao frio, em diferentes patologias dolorosas, como dor inflamatória e neuropática. Todas estas características deste canal tem identificado-o como um relevante alvo molecular para a descoberta de novos analgésicos. A ativação do canal TRPA1 por agonistas endógenos ou exógenos induz dor espontânea e inflamação neurogênica em modelos de nocicepção em roedores e também em humanos (ANDRADE; MEOTTI; CALIXTO, 2012; BAUTISTA; PELLEGRINO; TSUNOZAKI, 2013; NILIUS; APPENDINO; OWSIANIK, 2012).

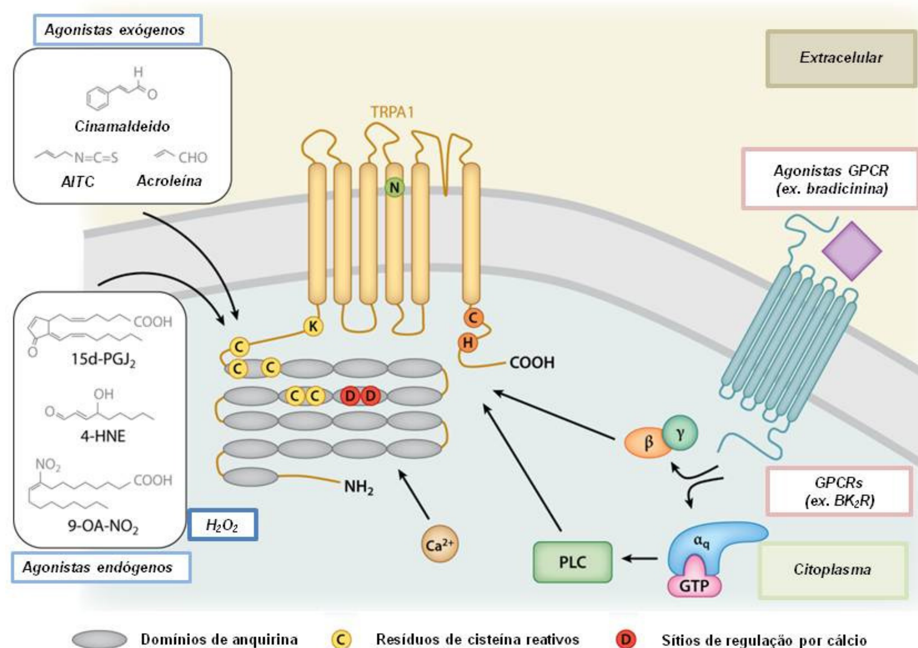

Figura 4 - Mecanismos de ativação e sensibilização do receptor TRPA1. Adaptado de Bautista, Pellegrino e Tsunozaki (2013).

Mesmo que o receptor TRPA1 tenha sido primeiramente identificado como um sensor ao frio nocivo (STORY et al., 2003), este evento ainda é um fator de discussão, porém a participação deste canal na hipersensibilidade ao frio observada após lesão tecidual ou após a dor neuropática, é uma característica já descrita em diversos estudos (DEL CAMINO et al., 2010). O desenvolvimento de hipersensibilidade ao frio observada em situações inflamatórias pode ser reduzida pela administração de antagonistas para o receptor ou ainda por indução da diminuição da sua síntese (DA COSTA et al., 2010; PETRUS et al., 2007). Além disso, já foi observado que o receptor TRPA1 poderia estar envolvido no aumento da sensibilidade à estímulos mecânicos em condições dolorosas. Tanto a sensibilização ao frio quando à estímulos mecânicos mediadas pelo receptor TRPA1 ainda tem mecanismos pouco esclarecidos, porém fatores como aumento da expressão do receptor e sensibilização por mediadores inflamatórios poderiam contribuir com estes mecanismos (ANDRADE; MEOTTI; CALIXTO, 2012; BAUTISTA; PELLEGRINO; TSUNOZAKI, 2013; NILIUS; APPENDINO; OWSIANIK, 2012). Em condições inflamatórias é possível observar um aumento da expressão do canal TRPA1 em locais de lesão ou até mesmo no GRD, um evento que é associado também a infiltração de diferentes células ao tecido afetado e a produção de mediadores inflamatórios, o que pode modular a atividade do receptor nestas condições (DA COSTA et al., 2010).

O receptor TRPA1 já foi identificado como um provável alvo para o desenvolvimento de analgésicos para o tratamento de diversas condições inflamatórias dolorosas, como por exemplo, dor pós-cirúrgica, cistite e artrite (ANDRADE; MEOTTI; CALIXTO, 2012; BAUTISTA; PELLEGRINO; TSUNOZAKI, 2013; NILIUS; APPENDINO; OWSIANIK, 2012). Sendo que já foi evidenciada a participação do receptor TRPA1 no desenvolvimento de dor inflamatória crônica em modelos de monoartrite induzida por CFA (adjuvante completo de Freund) ou osteoartrite induzida por monoiodoacetato (FERNANDES et al., 2011; MCGARAUGHTY et al., 2010). Além disso, o canal TRPA1 também pode estar envolvido nas respostas dolorosas induzidas por TNF-α, um mediador importante na inflamação e dor observada em artrite, e estes mecanismos seriam mediados por substâncias oxidantes produzidas e capazes de ativar o receptor TRPA1 (FERNANDES et al., 2011).

Este alvo molecular também foi evidenciado como um relevante mediador na hiperalgesia observada em modelos de dor neuropática, tanto em modelos induzidos por trauma como por administração de quimioterápicos (ANDRADE; MEOTTI; CALIXTO, 2012; BAUTISTA; PELLEGRINO; TSUNOZAKI, 2013; NILIUS; APPENDINO; OWSIANIK, 2012; TREVISAN et al., 2013b). Os mecanismos moleculares para a ativação e sensibilização do receptor TRPA1 ainda não são bem conhecidos, porém a principal hipótese seria que substâncias oxidantes produzidas durante estes estados dolorosos poderiam sensibilizar o receptor de maneira a induzir o desenvolvimento de hiperalgesia mecânica e ao frio (MATERAZZI et al., 2012; NASSINI et al., 2011; TREVISAN et al., 2013b). O receptor TRPA1 também parece estar envolvido na patofisiologia da dor observada na enxaqueca, sendo que diversos agonistas TRPA1 são capazes de induzir dores de cabeça. Além disso, um importante neuropeptídeo liberado após a ativação do receptor TRPA1, o CGRP, é um dos mediadores mais estudados nesta patologia debilitante (BENEMEI et al., 2013; MATERAZZI et al., 2013). Além disso, até o momento, não foi ainda relatado nenhum efeito adverso de antagonistas do receptor TRPA1 em modelos animais, então estes compostos parecem ser bons protótipos de novas drogas analgésicas (ANDRADE; MEOTTI; CALIXTO, 2012; BAUTISTA; PELLEGRINO; TSUNOZAKI, 2013; NILIUS; APPENDINO; OWSIANIK, 2012).

Concluindo, devido às diversas evidências mostrando a participação do canal TRPA1 em patologias dolorosas de diversas etiologias, e observando que mecanismos propostos para a patofisiologia da gota poderiam induzir a ativação deste canal, neste estudo avaliamos o papel do receptor TRPA1 na dor e inflamação observada em um modelo de ataques agudos de gota em roedores.

2 OBJETIVOS

2.1 Objetivo Geral

Investigar o envolvimento do receptor TRPA1 no desenvolvimento de nocicepção e inflamação em modelos de ataque agudo de gota em ratos e camundongos.

2.2 Objetivos Específicos

a. Observar a participação do receptor TRPA1 no desenvolvimento de nocicepção e inflamação nos modelos de ataque agudo de gota induzidos por administração de MSU intraplantar (i.pl.) ou intra-articular (i.a.) de cristais de MSU;

b. Verificar se a administração de MSU é capaz de alterar do conteúdo do receptor TRPA1 nos ratos submetidos a modelos de ataque agudo de gota;

c. Avaliar a participação de fibras sensoriais TRPV1 e TRPA1 positivas na nocicepção e inflamação induzidas pelos cristais de MSU em ratos;

d. Observar se os cristais de MSU ativam diretamente o receptor TRPA1 em neurônios sensoriais;

e. Caracterizar o H_2O_2 como o mediador endógeno da nocicepção e inflamação induzidas pelos cristais de MSU através da ativação do receptor TRPA1 em ratos.

3 ARTIGOS

Os resultados inseridos nesta tese apresentam-se sob a forma de artigos científicos, os quais se encontram aqui estruturados. Os itens Introdução, Materiais e Métodos, Resultados, Discussão dos Resultados e Referências Bibliográficas, encontram-se nos artigos. O primeiro artigo está disposto conforme aceito para publicação na revista Arthritis & Rheumatism, e o segundo artigo está estruturado como aquele enviado para publicação na revista Annals of the Rheumatic Diseases.

3.1 Artigo 1

Full Length

Arthritis & Rheumatism
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Running head: TRPA1 receptor activation mediates MSU inflammation

Title: TRPA1 receptor stimulation by hydrogen peroxide is critical to trigger pain during MSU-induced inflammation

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Abstract

Objective: Gout is a common cause of inflammatory arthritis provoked by the accumulation of monosodium urate (MSU) crystals, but the underlying mechanisms of the pain in acute gout attacks are poorly understood. The aim of the present study was to evaluate the role of transient receptor potential ankyrin 1 (TRPA1) and TRPA1 stimulants, such as hydrogen peroxide (H_2O_2), in the MSU-induced inflammation model in rodents.

Methods: MSU or H_2O_2 were injected into the hind paw of rodents, or applied in cultured sensory neurons and intracellular calcium *in vitro* and inflammatory or nociceptive responses *in vivo* were evaluated. Pharmacological, genetic or biochemical tools and methods have been used.

Results: We found that TRPA1 antagonism, TRPA1 gene deletion or defunctionalization by capsaicin pretreatment of peptidergic TRP-expressing primary sensory neurons markedly decreased MSU-induced nociception and edema. In addition to these neurogenic effects, MSU increased H_2O_2 levels in the injected tissue, an effect that was abolished by the H_2O_2-detoxifing enzyme, catalase. H_2O_2, but not MSU, directly stimulated sensory neurons through the activation of TRPA1. Nociceptive responses evoked by MSU or H_2O_2 injection were attenuated by catalase, the reducing agent dithiothreitol. In addition, MSU injection increased the expression of TRPA1 and TRPV1 as well as enhanced cellular infiltration and IL-1β levels, which were blocked by TRPA1 antagonism.

Conclusion: Our results suggest that MSU-injection increases tissue H_2O_2 thereby stimulating TRPA1 on sensory nerve endings to produce inflammation and nociception. TRPV1, by a hitherto unknown mechanism, also contributes to these responses.

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Introduction

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

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

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

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

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

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Materials and Methods

Animals

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

Drugs

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

Preparation and administration of MSU crystals

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

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Evaluation of nociceptive response

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

Determination of inflammatory response

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

Treatment protocols

HC-030031 (TRPA1 selective antagonist, 30-300 nmol/paw), camphor (TRPA1 poorly-selective antagonist, 150 nmol/paw), SB-366791 (TRPV1 selective antagonist, 10 nmol/paw), indomethacin (cyclooxygenase inhibitor, 280 nmol/paw) or their vehicle solution (0.1% DMSO in PBS, 100 µL/paw,) were s.c. co-injected with MSU (0.25 mg/paw), its vehicle (PBS, 100 µL), or the TRPA1 agonists allyl isothiocyanate (AITC, 1 nmol/paw) and H_2O_2 (3 µmol/paw). In another set of experiments, we have orally (p.o.)

administrated the HC-030031 (300 µmol/kg) or its vehicle (1% DMSO in PBS, 1 mL/kg) 1 hour before the s.c. injection of MSU (0.25 mg/paw) or its vehicle (PBS, 100 µL). Moreover, we have co-injected the SB-366791 (0.1 nmol/paw) plus HC-030031 (30 nmol/paw) or their vehicle (100 µL/paw, 0.1% DMSO in PBS) with MSU (0.25 mg/paw), H_2O_2 (3 µmol/paw), or its vehicle (PBS, 100 µL). In a different set of experiments, catalase from bovine liver (300 UI/paw), DTT (20 nmol/paw), or their vehicle (PBS, 100 µL/paw) were co-injected with MSU (0.25 mg/paw), H_2O_2 (3 µmol/paw), AITC (1 nmol/paw, co-injected only with catalase or vehicle) or its vehicle (PBS, 100 µL). DTT (20 nmol/paw) was injected 5 minutes before the H_2O_2 to prevent any ongoing reaction with H_2O_2. External hind paw temperature was measured before and 10 minutes after the i.pl. injection of H_2O_2 (3 µmol/paw) as described previously (26).

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

Western blot analysis

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

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

Calcium influx in rat dorsal root ganglia (DRG) neurons

Rat DRG neurons were cultured as previously described (23). Intracellular calcium fluorescence was measured in neurons, as previously reported (21,23). Neurons were exposed to uric acid (100-300 µM), MSU crystals (0.003-0.100 g/L), H_2O_2 (10-5000 µM), acrolein (30 µM), capsaicin (0.1 µM), or their vehicles (buffer solution). HC-030031 and SB-366791 vehicles (used in all the in vitro experiments) were both 1% DMSO. Results are expressed as the increase of $Ratio_{340/380}$ over the baseline, normalized to the maximum effect induced by ionomycin (5 µM) added at the end of the experiment (% Change $R_{340/380}$).

H_2O_2 production assay

To determine the H_2O_2 levels in paw skin after MSU s.c. injection, we performed a protocol using the phenol red-HRPO method (31). Briefly, 0.25 to 48 hours after MSU (0.25 mg/paw) or vehicle (PBS, 100 µL), and 0.25 hours after MSU or vehicle plus HC-

030031 (300 nmol/paw), SB-366791 (10 nmol/paw), or catalase (300 UI/paw) injection, rats were euthanized and hind paw skins were removed. Basal values were assessed in rats not injected. The samples were homogenized in 50 mM phosphate buffer (pH 7.4) containing 5 mM of sodium azide at 4°C for 60 seconds, and the homogenate was centrifuged (12,000 x*g*, 20 minutes, 4°C. The supernatant obtained was used to determine the H_2O_2 levels (31). The H_2O_2 levels were expressed as µmol of H_2O_2 on the basis of a standard curve of HRPO-mediated oxidation of phenol red by H_2O_2, corrected by protein content (in mg) of the paw skin sample analyzed.

Statistical analysis

All values are expressed as mean ± S.E.M. ED_{50} values (i.e., the necessary dose of H_2O_2 to elicit 50% of the response relative to the control value), which are reported as geometric means accompanied by their respective 95% confidence limits. The percentages of inhibition are reported as the mean ± SEM and calculated with the maximum developed responses obtained after injection of MSU, AITC or H_2O_2 when compared to vehicle-treated animals (control). The statistical significance between groups was assessed by the Student's "t" test (to evaluate statistical significance between 2 groups), in addition to 1- (to assess statistical significance among more than 2 groups) or 2- (to evaluate statistical significance among 2 or more groups in time-course curves) way analysis of variance (ANOVA) when appropriate. Bonferroni's post hoc test was conducted when 1- or 2-way ANOVA was used. *P* values lower than 0.05 ($P < 0.05$) were considered to be significant. The ED_{50} values were determined by nonlinear regression analysis with a sigmoid dose-response equation using GraphPad Software version 5.0 (GraphPad Software Inc, La Jolla, CA, USA).

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Results

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

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

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

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

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

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

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

Inflammatory responses induced by MSU injection were reduced by TRPA1 blockage

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

Accepted Article

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

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

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

MSU induces H_2O_2 production to stimulate TRPA1 and trigger nociception and edema

Since MSU stimulates ROS production and TRPA1 is a sensor of oxidative stress (6, 32), we tested whether ROS were involved in TRPA1-medited responses evoked by MSU. As catalase decomposes H_2O_2 to H_2O_2 and O_2 (33), we co-injected the enzyme with MSU. Catalase (300 UI/paw) abolished the development of ongoing nociception (100% inhibition), cold allodynia (100% inhibition) and edema (95% inhibition) (Figure 4A). However, the ongoing nociception, cold allodynia, and edema induced by AITC s.c. paw injection were not reduced by catalase co-administration (data not shown). Next, we found an increase in H_2O_2 production in the injected tissue 0.25 to 6 hours after MSU

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

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

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

Accepted Article

nociception (81% inhibition), cold allodynia (100% inhibition), and edema (100% inhibition) (Figure 5D). Moreover, H_2O_2 (s.c. paw injection) increased external paw skin temperature (from 28±1ºC before treatment to 32±0.8ºC 10 minutes after H_2O_2 injection, $P < 0.05$, n = 5-6), an effect that could contribute to TRPV1 activation/sensitization (27,35).

It has been demonstrated that reactive TRPA1 agonists bind to intracellular cysteine residues to activate the channel, an effect prevented by the reducing agent DTT, which reverses cysteine disulfide formation and nitrosylation or oxidization of cysteine sulfhydryls (36,37). Local pre-treatment with DTT (20 nmol/paw) decreased both MSU and H_2O_2-induced ongoing nociception (71% and 100% inhibition), cold allodynia (100% and 100% inhibition), and edema (90% and 100% inhibition) (Figure 4D), thus supporting the participation of cysteine residues of TRPA1 receptor in this phenomenon.

Accepted Article

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Discussion

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

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

Accepted Article

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

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

Stimulation of resident or infiltrating proinflammatory cells by MSU crystals and uric acid is known to generate ROS (10-12). We found that MSU injection concomitantly to the appearance of nociception and edema induced a remarkable increase in H_2O_2 levels within the injected tissue. We have detected that the increase of MSU-induced at the H_2O_2 concentration peaked at 0.25 hour and it was still significantly different from vehicle up to 6 hours, but in lower levels. Thus, H_2O_2 levels seem to be pivotal to the early nociception

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

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

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

hypothesis is supported by the finding that H_2O_2 injection increased paw temperature by about 4ºC, a phenomenon which, in turn, could lead to gate TRPV1 (27,35). TRPV1 sensitization by H_2O_2 (48) and the ensuing enhanced stimulation by heat might also exaggerate TRPA1 activation, as observed in previous (27,35,49) and present studies. Thus, it is possible that, it has been shown in other experimental conditions of inflammatory pain (16-18), both TRPV1 and TRPA1 contribute synergistically to the development of inflammatory painful responses evoked by MSU.

In conclusion, H_2O_2 production by resident cells and the consequent activation of TRPA1 receptor in sensory neurons seem to start the process that generates MSU-induced pain and inflammation. From this initial event, additional mechanisms contributing to the overall inflammatory and sensory response are progressively recruited, in a time-dependent manner. Accordingly, early blockade of TRPA1 in gout might be a reliable pharmacological choice to completely suppress inflammation and pain in acute gout attacks.

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Accepted Article

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Author Contributions

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

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

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Accepted Article

Figure legends

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

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

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

Figure 3. Monosodium urate (MSU) crystals-induced inflammatory responses were reduced by TRPA1 antagonism. (A) Myeloperoxidase (MPO) level, (B) IL-1β content, or (C) number of inflammatory infiltrated cells per high power fields (HPF, 20x) was increased 6 hours after MSU (0.25 mg/paw) injection, and these effects were reduced by the co-administration of HC-030031 (HC, 300 nmol/paw) or indomethacin (INDO, 280 nmol/paw). (D) Typical traces and pooled data showing that neither MSU (0.1 mg/ml) or Uric Acid (300 µM) produced a calcium influx in rat capsaicin-sensitive DRG neurons that normally respond to ACR or H_2O_2. HC-030031 (HC, 30 µM) significantly reduced the effect evoked by ACR (30 µM) or H_2O_2 (500 µM). Veh is the vehicle of the various agonist (buffer), Veh1 is the vehicle of the antagonists (1% DMSO). N.D. (not detectable). Each column represents the mean ± S.E.M. of five to seven samples or value of at least 25 neurons.The asterisks denote the significance levels. $^{***}P< 0.001$ in comparison to vehicle treated group (pre-treated with vehicle) or vehicle (Veh or Veh1); or $^{\#\#}P< 0.01$, $^{\#\#\#}P< 0.001$ difference in comparison to MSU treated group (pre-treated with vehicle); 1-way ANOVA followed by Bonferroni's post hoc test.

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Figure 4. MSU induces H_2O_2 production to stimulate TRPA1 and trigger nociception and edema. (A) MSU (0.25 mg/paw)-induced responses were reduced by the catalase (300 UI/paw) s.c. paw administration. (B) The MSU (0.25 mg/paw) is able to induce the H_2O_2 production in skin paw at 0.25 to 6 hours after s.c. paw administration. (C) The injection of catalase (300 UI/paw), but not HC-030031 (HC, 300 nmol/paw) or SB-366791 (SB, 10 nmol/paw), was able to reduced the H_2O_2 content (0.25 hour after injection). (D) MSU (0.25 mg/paw) and H_2O_2 (3 µmol/paw) elicited ongoing nociception, cold allodynia, and edema were reduced by the cell-permeable reducing agent dithiothreitol (DTT, 20 nmol/paw) s.c. paw injection. Ongoing nociception, cold allodynia, and edema were measure from 0-10 minutes, 0.25 hour, and 0.5 hour after injection, respectively. Each column represents the mean ± S.E.M. of five to seven rats. The asterisks denote the significance levels. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ in comparison to vehicle (Veh) treated group; or $^{\#}P < 0.05$, $^{\#\#\#}P < 0.001$ difference in comparison to MSU or H_2O_2 treated group; 1-way ANOVA followed by Bonferroni's post hoc test.

Figure 5. H_2O_2-mediated responses were dependent on TRPA1 activation. (A) Time course of H_2O_2 (3 µmol/paw) elicited ongoing nociception, cold allodynia, and edema in rats. (B) Dose-response curve of ongoing nociception, cold allodynia, and edema induce by H_2O_2 (0.3, 1, 3, or 10 µmol/paw). (C) TRPA1 antagonists reduced the responses induced by H_2O_2 s.c. paw injection. The selective antagonist HC-030031 (HC, 300 nmol/paw), or the non-selective TRPA1 antagonist camphor (Cam, 150 nmol/paw), the selective TRPV1 antagonist SB-366791 (SB, 10 nmol/paw), or vehicle (Veh) were s.c. co-injected with H_2O_2 (3 µmol/paw). (D) The ongoing nociceptive, cold allodynic, and edematogenic responses elicited by H_2O_2 (3 µmol/paw) were largely reduced by local administration of HC-030031 (HC, 30 nmol/paw) jointly to SB-366791 (SB, 0.1 nmol/site) in a subdose manner. Ongoing nociception, cold allodynia, and edema were measure (in B,

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Tables

Table 1. Controls for the pharmacological treatments.

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

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

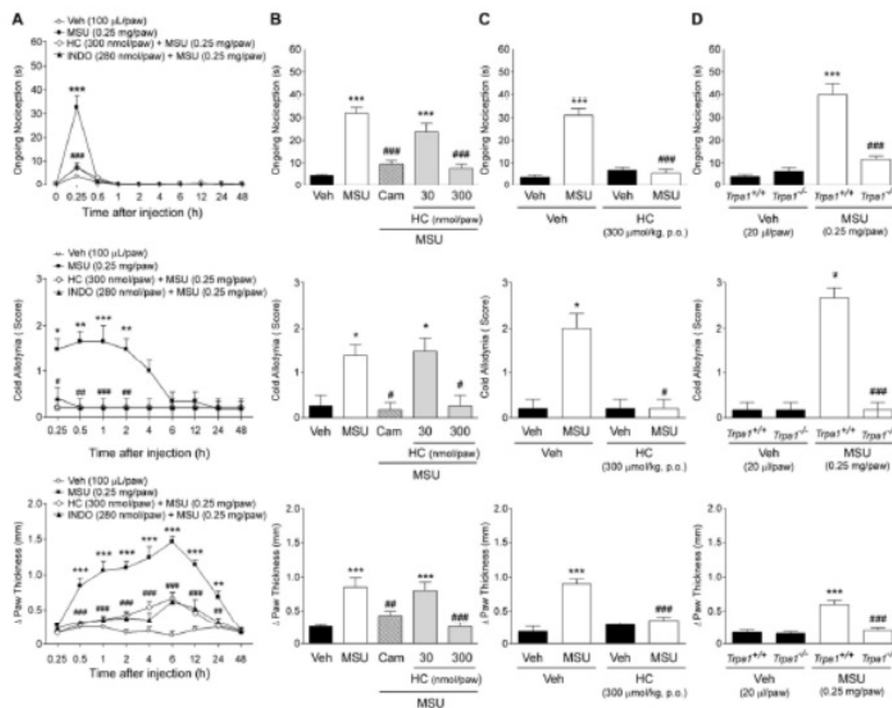

Figure 1. TRPA1 activation mediates monosodium sodium (MSU) crystals-induced ongoing nociception, cold allodynia, and edema in rodents. (A) MSU (0.25 mg/paw)-induced responses were largely reduced by local administration of the TRPA1 receptor selective antagonist HC-030031 (HC, 300 nmol/paw) or indomethacin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

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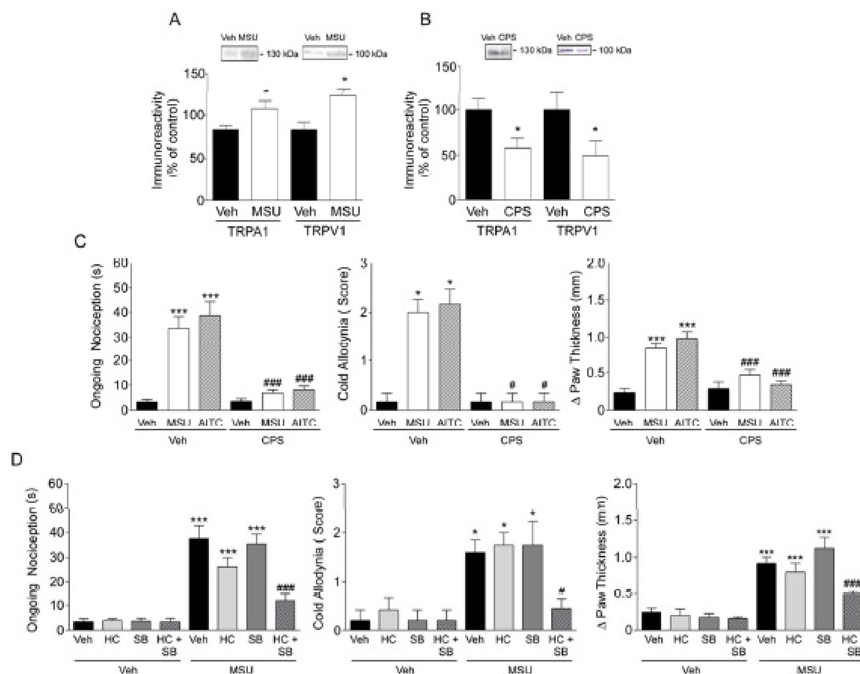

Figure 2. Monosodium sodium (MSU) crystals injection increased TRPA1 and TRPV1 expression, and ablation of positive TRPA1 and TRPV1 fibers diminished the MSU-elicited responses. Western blot (inset) showing TRPA1 or TRPV1 immunoreactivity in (A) skin hindpaw samples 6 hours after the injection of MSU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

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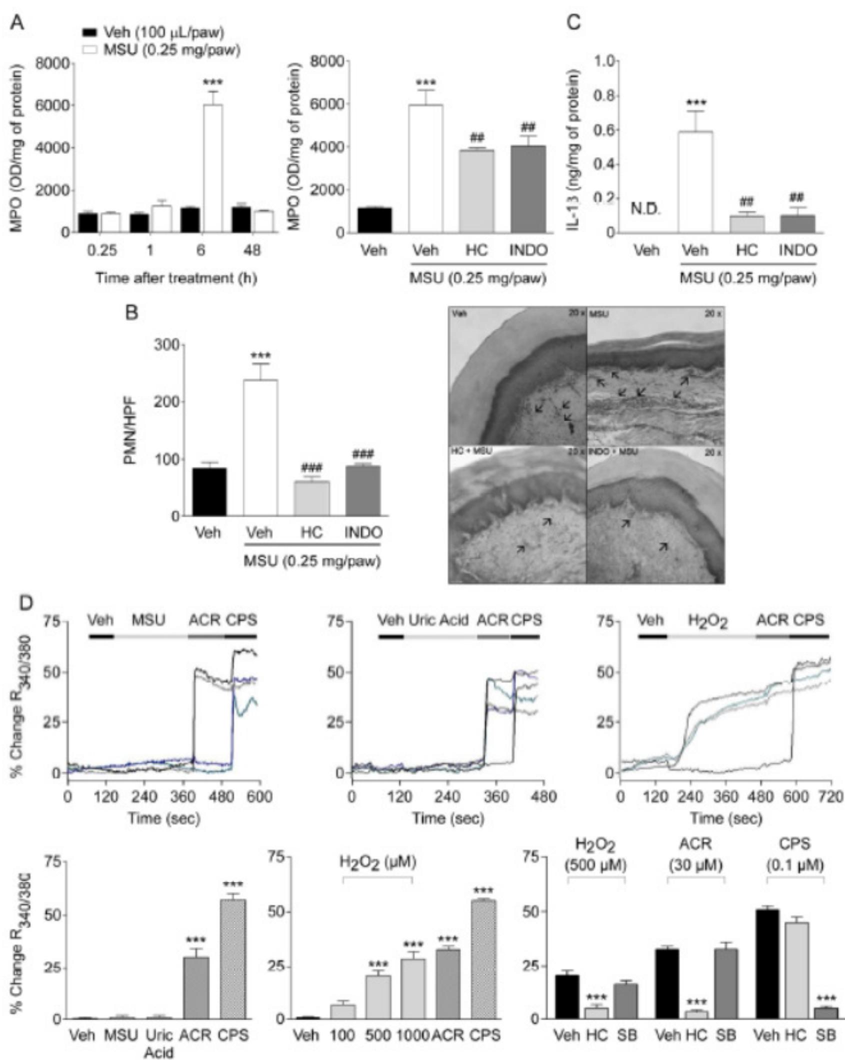

Figure 3. Monosodium urate (MSU) crystals-induced inflammatory responses were reduced by TRPA1 antagonism. (A) Myeloperoxidase (MPO) level, (B) IL-1β content, or (C) number of inflammatory infiltrated cells per high power fields (HPF, 20x) was increased 6 hours after MSU (0.25 mg/paw) injection, and these effects were reduced by the co-administration of HC-030031 (HC, 300 nmol/paw) or indomethacin (INDO, 280 nmol/paw). (D) Typical traces and pooled data showing that neither MSU (0.1 mg/ml) or Uric Acid (300 µM) produced a calcium influx in rat capsaicin-sensitive DRG neurons that normally respond to ACR or H2O2. HC-030031 (HC, 30 µM) significantly reduced the effect evoked by ACR (30 µM) or H2O2 (500 µM). Veh is the vehicle of the various agonist (buffer), Veh1 is the vehicle of the antagonists (1% DMSO). N.D. (not detectable). Each column represents the mean ± S.E.M. of five to seven samples or value of at least 25 neurons.The asterisks denote the significance levels. ***$P < 0.001$ in comparison to vehicle treated group (pre-treated with vehicle) or vehicle (Veh or Veh1); or ##$P < 0.01$, ###$P < 0.001$ difference in comparison to MSU treated group (pre-treated with vehicle); 1-way ANOVA followed by Bonferroni's post hoc test. 144x181mm (300 x 300 DPI)

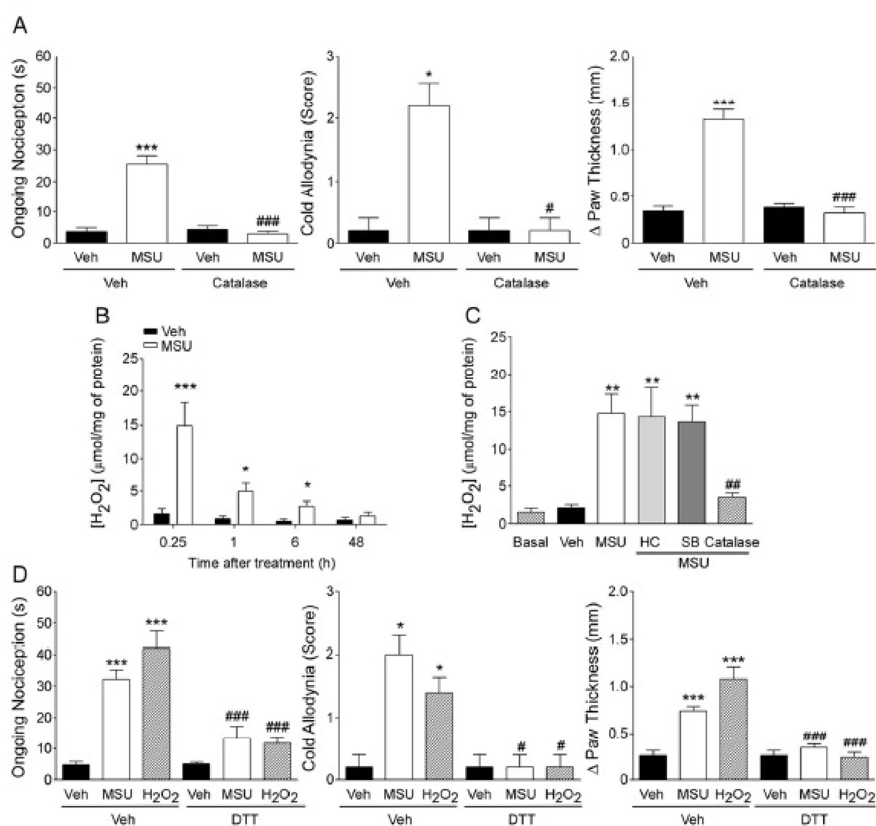

Figure 4. MSU induces H2O2 production to stimulate TRPA1 and trigger nociception and edema. (A) MSU (0.25 mg/paw)-induced responses were reduced by the catalase (300 UI/paw) s.c. paw administration. (B) The MSU (0.25 mg/paw) is able to induce the H2O2 production in skin paw at 0.25 to 6 hours after s.c. paw administration. (C) The injection of catalase (300 UI/paw), but not HC-030031 (HC, 300 nmol/paw) or SB-366791 (SB, 10 nmol/paw), was able to reduced the H2O2 content (0.25 hour after injection). (D) MSU (0.25 mg/paw) and H2O2 (3 µmol/paw) elicited ongoing nociception, cold allodynia, and edema were reduced by the cell-permeable reducing agent dithiothreitol (DTT, 20 nmol/paw) s.c. paw injection. Ongoing nociception, cold allodynia, and edema were measure from 0-10 minutes, 0.25 hour, and 0.5 hour after injection, respectively. Each column represents the mean ± S.E.M. of five to seven rats. The asterisks denote the significance levels. *P< 0.05, **P< 0.01, ***P< 0.001 in comparison to vehicle (Veh) treated group; or #P< 0.05, ###P< 0.001 difference in comparison to MSU or H2O2 treated group; 1-way ANOVA followed by Bonferroni's post hoc test.
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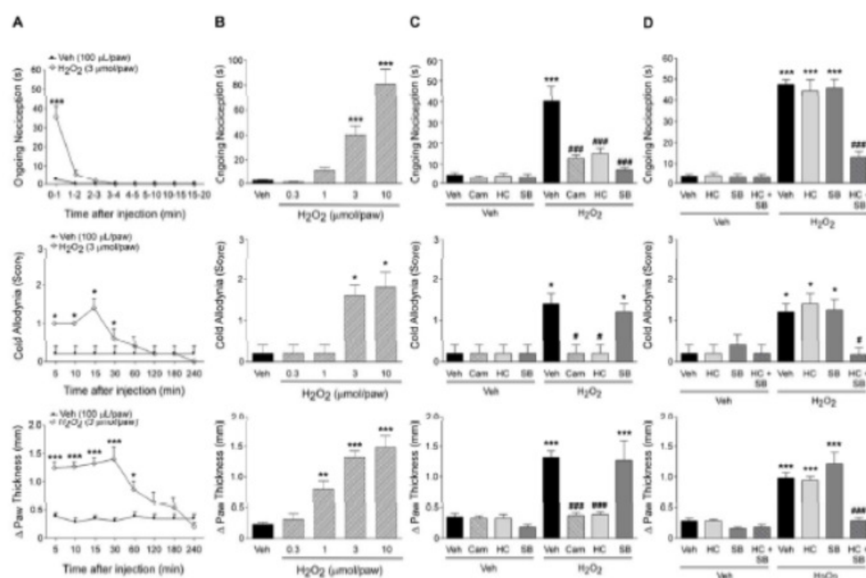

Figure 5. H2O2-mediated responses were dependent on TRPA1 activation. (A) Time course of H2O2 (3 μmol/paw) elicited ongoing nociception, cold allodynia, and edema in rats. (B) Dose-response curve of ongoing nociception, cold allodynia, and edema induce by H2O2 (0.3, 1, 3, or 10 μmol/paw). (C) TRPA1 antagonists reduced the responses induced by H2O2 s.c. paw injection. The selective antagonist HC-030031 (HC, 300 nmol/paw), or the non-selective TRPA1 antagonist camphor (Cam, 150 nmol/paw), the selective TRPV1 antagonist SB-366791 (SB, 10 nmol/paw), or vehicle (Veh) were s.c. co-injected with H2O2 (3 μmol/paw). (D) The ongoing nociceptive, cold allodynic, and edematogenic responses elicited by H2O2 (3 μmol/paw) were largely reduced by local administration of HC-030031 (HC, 30 nmol/paw) jointly to SB-366791 (SB, 0.1 nmol/site) in a subdose manner. Ongoing nociception, cold allodynia, and edema were measure (in B, C, and D) from 0-10 minutes, 0.25 hour, and 0.5 hour after injection, respectively. Each column represents the mean ± S.E.M. of five to nine rats. The asterisks denote the significance levels. *P< 0.05, **P< 0.01, ***P< 0.001 in comparison to vehicle treated group; or #P<0.05, ###P< 0.001 difference in comparison to H2O2 treated group; 1 or 2-way ANOVA followed by Bonferroni's post hoc test.
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Acce

3.2 Artigo 2

TRPA1 receptor activation mediates hyperalgesia and inflammation in a model of acute gout in rodents

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Keywords: gout; nociception; hydrogen peroxide; IL-1β; CGRP.

Word count: 2932 words

ABSTRACT

Objectives: Acute gout attacks produce severe joint pain and inflammation associated with monosodium urate (MSU) crystals deposits in the joints and oxidative stress production. The transient potential receptor ankyrin 1 (TRPA1) is expressed by a subpopulation of peptidergic sensory fibers and contributes to pain and inflammation via activation by endogenous oxidants, including hydrogen peroxide (H_2O_2). The aim of the present study was to investigate the role of TRPA1 in hyperalgesia and inflammatory responses in an articular model of acute gout attack in rodents.

Methods: Inflammatory parameters and mechanical hyperalgesia were measured in male Wistar rats, wild-type (*Trpa1*$^{+/+}$) or TRPA1-deficient (*Trpa1*$^{-/-}$) male mice, the latter of which received intra-articular (i.a., ankle) injection of MSU. The role of TRPA1 was assessed by receptor antagonism, gene deletion or expression, sensory fiber defunctionalization, and calcitonin gene-related peptide (CGRP) release.

Results: We confirmed that nociceptor defunctionalization, TRPA1 antagonist treatment (via i.a. or oral administration), and TRPA1 gene ablation abated hyperalgesia and certain inflammatory parameters (edema, H_2O_2 generation, IL-1β release and neutrophil infiltration) induced by i.a. MSU injection. In the i.a. model, we also confirmed that MSU generated H_2O_2 thereby activating TRPA1. In addition, we showed that in this i.a. model of acute gout, MSU evoked TRPA1-dependent both CGRP release and plasma protein extravasation.

Conclusions: TRPA1 activation by MSU challenge-generated H_2O_2 mediates the entire inflammatory response in an acute gout rodent model, thus strengthening the role of TRPA1 as a potential target for treatment of acute gout attacks.

INTRODUCTION

Gout is the principal cause of inflammatory arthritis in men and postmenopausal women. The initial discovery of monosodium urate (MSU) crystals in the joints of gout patients led to the clinical definition of gout as an inflammatory arthritic disease. Acute attacks of gout are accompanied by severe joint pain and articular/periarticular inflammation, which is associated with neutrophil infiltration and production of pro-inflammatory cytokines (mainly interleukin-1β).[1, 2]

Regular control of serum urate levels, using allopurinol and febuxostat, and the reduction of the incidence of acute gout burdens, by nonsteroidal anti-inflammatory drugs or colchicine, are the most popular therapies for gout, which, however may cause significant adverse effects, thus limiting their use. Although new alternatives have been discovered, such as the modified uricases (pegloticase) and interleukin-1 inhibitors,[1, 3, 4] gout patients are still undertreated, and novel strategies for the relief of acute gout attacks with a good efficacy and safety profile are required. In this view, a validation of new targets for the treatment of acute gout attack is urgently needed.

Previous findings showed that oxidant substances are produced during the process that results in acute gout attack.[1, 5] Accordingly, it has been found that the antioxidant vitamin C affords some beneficial effects on gout attacks.[6-8] Thus, the hypothesis that oxidants compounds, by still-unknown mechanisms, could trigger acute gout attacks has been advanced. The transient receptor potential ankyrin 1 (TRPA1) is a non-selective cation channel activated by oxidant substances, such as hydrogen peroxide (H_2O_2).[9] TRPA1 is co-expressed in sensory neurons along with the hot chili pepper receptor TRP vanilloid 1 (TRPV1) and the vasodilator and pro-inflammatory mediator calcitonin gene-related peptide (CGRP). Independent preclinical studies using different rodent models showed that TRPA1 antagonists inhibit nociception and inflammation.[10-12]

Previously, we found that H_2O_2 production and subsequent TRPA1 activation contribute significantly to painful and inflammatory responses induced by MSU subcutaneous injection.[13] However, the role of TRPA1 in the development of the inflammatory response by MSU crystal injection into the articular tissue, which different from subcutaneous tissue, represents a reliable model of acute gout attack,[14, 15] has not been examined. The aim of the present study was to

investigate the role of TRPA1 and H_2O_2 in the mechanical hyperalgesia and inflammatory responses in an articular model of acute gout attack in rodents.

MATERIALS AND METHODS

Ethical statement

All experiments were carried out according to the current guidelines for the care of laboratory animals (European Communities Council (ECC) guidelines for animal care procedures and the Italian legislation (DL 116/92) application of the ECC directive 86/609/EEC) and ethical guidelines for investigations of experimental pain in conscious animals.[16] All protocols were also approved by the Ethics Committees of the Federal University of Santa Maria (process number 108/2011(2) and the University of Florence (research permit ♯143/2008-B and ♯204/2012-B). To describe the behavioral studies, we have followed the ARRIVE guidelines.[17]

Animals

Experiments were performed using adult male Wistar rats (200-250 g, bred in our vivarium) and littermate wild-type (*Trpa1*$^{+/+}$) or TRPA1-deficient (*Trpa1*$^{-/-}$; B6;129P-Trpa1tm1Kykw/J, 20-30 g) mice were generated by crossing heterozygous animals on a C57BL/6 background (Jackson Laboratories, Bar Harbor, Maine, USA).[18] Animals were housed in a controlled-temperature environment in individually ventilated rat or mouse cages (5 per cage for rats, 10 per cage for mice with wood shaving bedding and no environment enrichment) maintained at 22±1℃. Animals were maintained with a 12 hours light/dark cycle (lights on from 6:00 a.m. to 6 p.m.) and fed with rodent chow (Puro Lab 22 PB pelleted form, Puro Trato, Rio Grande do Sul, Brazil for rats or Global Diet 2018, Harlan, Lombardia for mice) and tap water *ad libitum*. Before experiments, animals were allowed to acclimatize to the experimental room for at least 1 hour and to their housing environment for at least 72 hours after arrival.

Drugs

Unless otherwise indicated, all reagents were from Sigma (Sigma, St Louis, MO, USA) and were dissolved in the appropriate vehicle solutions. The TRPA1-selective antagonist HC-030031 was synthesized as previously described.[19]

Study design

The primary outcome in the behavioral experiments was mechanical hyperalgesia, and the secondary outcome was edema formation after the i.a. injection of MSU or H_2O_2. These responses were evaluated in the same group of animals for all the treatments. For behavioral experiments, we used a group size of six rats (or six samples) or seven mice for all tests. The group size for each experiment was determined by sample size estimation [20] (ANOVA sample sizes, desired power 0.8, $\alpha = 0.05$, standard deviation = 4.5 and difference to detect = 6.5 for rats, or standard deviation = 0.25 and difference to detect = 0.35 for mice) for each experiment, based on previous results obtained in our laboratory, where we have observed mechanical hyperalgesia after MSU i.a. injection. Each experiment was repeated 2 to 3 times. Allocation concealment was not performed before the i.a. injection because we had allocated the animals in different groups to yield groups with similar basal values in the initial phase of the experiment. Experimenters were blinded to the genotype and the drug treatment when performing the tests and to the experimental group when performing analysis. The inclusion and exclusion criteria for the behavioral test was the development of mechanical hyperalgesia and edema formation that were changed at least 30% compared with the baseline values. No animal or sample was excluded from the analysis. Experiments were conducted between 8:00 a.m. and 5:00 p.m.

Procedures for MSU i.a. injection and behavioral experiments

Intra-articular injection of MSU crystals

An endotoxin-free MSU crystal suspension (with a mean length of 12 ± 2 μm),[21] vehicle, or drugs in a volume of 50 or 20 μL (1.25 mg/site) for rats and mice, respectively, were injected into the medial side of the left tibiotarsal joint (ankle) under isoflurane anesthesia.[14, 15]

Mechanical hyperalgesia

Mechanical hyperalgesia, observed as an increase in nociceptive response, was assessed according to a previously reported procedure and expressed as 50% mechanical paw withdraw threshold (in g).[22, 23]

Edema formation

Edema formation was described as the difference (Δ) between the basal value and the test value measured using a digital caliper.[21]

Evaluation of inflammatory cell accumulation and measurement of calcitonin gene-related peptide (CGRP) and cytokine content

Hematoxylin-eosin (H&E) staining and histological evaluation of emigrated neutrophils was performed following the i.a. injection of MSU (1.25 mg/site) in articular tissue.[24] Furthermore, various inflammatory parameters were evaluated after MSU injection in synovial lavage samples.[25] The total number of cells was counted using a Neubauer chamber.[25] Myeloperoxidase (MPO) activity was determined as described previously.[26] Protein content in the synovial fluid was determined as described elsewhere.[27] We also measured the CGRP-like immunoreactivity (CGRP-LI) in the synovial lavage fluid as previously described using a commercial ELISA kit (Bertin Pharma, France).[28] Moreover, the synovial lavage fluid was also assayed for interleukin-1 beta (IL-1β) content using an ELISA kit (PeproTech Inc., Rocky Hill, NJ, USA).[13]

Procedures for drug treatment

Here, we observed the antinociceptive and anti-inflammatory effects of drugs using the time points of 1 and 4 hours after MSU injection. The 1-hour time point was chosen because, at that time, we observed all the nociceptive signs without cellular infiltration; however, at the 4-hour time point, we observed the nociceptive and inflammatory signs.[13] The selective and the poorly selective TRPA1 antagonists, HC-030031 (300 nmol/site) and camphor (150 nmol/site), respectively, or a vehicle solution (50 µL/site, 0.1% DMSO in PBS) were co-injected i.a. with MSU (1.25 mg/site), the TRPA1 agonists AITC (1 nmol/site), or H_2O_2 (3 µmol/site), or vehicle. In addition, we tested whether the systemic administration of HC-030031 (300 µmol/kg, p.o.) or vehicle (1% DMSO in PBS, 1 mL/kg, p.o.) 1 hour before the i.a. injection of MSU (1.25 mg/site) or vehicle (PBS, 50 µL) reduced MSU-mediated nociception and edema. In addition, MSU crystals (1.25 mg/site, 20 µL) or PBS (20 µL/site) were injected i.a. into *Trpa1*$^{-/-}$ and *Trpa1*$^{+/+}$ mice, and mechanical allodynia and edema were evaluated as described above.

To explore the considerable role of TRPA1-positive fibers in MSU-induced nociception and edema formation, we also employed an ablation protocol using an intraneural injection of capsaicin.[21, 13] Animals were used after 7 days to observe the responses to i.a. injection of MSU crystals (1.25 mg/site), AITC (1 nmol/site, a TRPA1 agonist used as a positive control), or vehicle (50 µL/site). In a different set of experiments, catalase (a H_2O_2-detoxifying enzyme) from bovine liver (300 UI/site), DTT (20 nmol/site) or vehicle (50 µL/site) were co-injected with MSU (1.25 mg/site), H_2O_2 (3 µmol/site) or vehicle (50 µL/site). The treatment time and drug doses were based on published data as well as on pilot experiments using positive controls (data not shown). Animals were sacrificed with a high dose of intraperitoneal (i.p.) sodium pentobarbital (200 mg/kg).

Western blot analysis

Western blot analysis was carried out as described previously.[13, 21] Ponceau staining served as a loading control. A specific anti-TRPA1 primary antibody (anti-TRPA1 polyclonal antibody; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) was used. The results were normalized to the control group densitometry values and expressed as the relative amount of TRPA1 immunoreactivity.

Determination of hydrogen peroxide levels

H_2O_2 content in synovial tissue was assessed after i.a. injection of MSU (1.25 mg/site) or vehicle (50 µL/site) at different time points (1 and 4 hours) using the phenol red-HRPO method.[13]

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

Briefly, rat knee synovial membrane was removed and assayed as described previously.[29] After a stabilization period (2 hours), tissues were incubated with MSU (25 mg/mL) or vehicle (assay buffer). Then, after different time points (0.25 to 4 hours), aliquots (100 µL) were removed for H_2O_2 measurement as described above. We also incubated the synovial membranes with colchicine (10 µM) or vehicle (assay buffer), and after 1 hour, the tissues were treated with MSU (25 mg/mL) or vehicle.[30]

Statistical analysis

All results were expressed as the mean ± S.E.M. Before performing statistical significance analysis, data were tested for normality using the Kolmogorov-Smirnov test and for homogeneity using the Bartlett test. Hyperalgesia data were log transformed to meet parametrical assumptions. The difference between 2 groups at one time point were analyzed by Student's t test; differences among 3 or more groups at one time point were analyzed by one-way analysis of variance (ANOVA) followed by Bonferroni's test; differences among 3 or more groups at different times were analyzed by two-way ANOVA followed by Bonferroni's test. Statistical analysis was performed using GraphPad Software 5.0 (GraphPad Software, San Diego, CA, USA). The percentage inhibition values were reported as the mean ± S.E.M. obtained in each experiment in relation to the control values. P values less than 0.05 ($P < 0.05$) were considered significant. To meet the ANOVA assumptions, the mechanical allodynia data were log transformed prior to statistical analysis.

RESULTS

MSU-induced edema and allodynia after i.a. injection is largely mediated by TRPA1 receptor activation

Before MSU injection, animals were healthy and without any detectable hyperalgesia or edema (data not shown). Local treatment with the TRPA1-selective antagonist HC-030031 (300 nmol/site, i.a.) or with the poorly selective TRPA1 antagonist camphor (150 nmol/site, i.a.) was able to decrease MSU-induced allodynia and also i.a. edema 1 and 4 hours after treatment (Fig. 1A-B). In addition, i.a. injection of the selective TRPA1 agonist AITC caused nociceptive and edematogenic responses, which were markedly reduced by the TRPA1 antagonists (HC-030031 or camphor, i.a.) (Table 1). Oral administration of HC-030031 (300 µmol/kg, p.o.) also largely reduced the development of MSU-elicited allodynia and edema formation from 1 to 4 hours after treatment (Fig. 1C-D). HC or camphor per se did not produce any measurable inflammatory response compared to vehicle (Fig. 1). *Trpa1*$^{+/+}$ mice showed mechanical allodynia and edema formation after i.a. injection of MSU at all evaluated time points. However, *Trpa1*$^{-/-}$ mice presented a marked reduction in MSU-trigged responses (Fig. 1E-F). Further, in naïve rats, ablation of

TRPA1-positive nerves by perineural injection of capsaicin reduced TRPA1 channel immunoreactivity in the synovial tissue (64% reduction) 7 days after treatment (Fig. 2A). In addition, ablation of TRPA1-positive fibers was associated with diminished MSU- and AITC-induced allodynia and edema from 1 to 4 hours (Fig. 2B-C).

Table 1. Controls for the pharmacological treatments.

Time after treatment	1 hour		4 hours	
Treatment	Mechanical Hyperalgesia (PWT in g)	Δ Paw Thickness (mm)	Mechanical Hyperalgesia (PWT in g)	Δ Paw Thickness (mm)
Vehicle (100 µL/site)	51±9	0.2±0.05	50±5	0.2±0.06
HC-030031 (300 nmol/site)	52±9	0.2±0.05	56±4	0.3±0.07
Camphor (150 nmol/site)	43±7	0.2±0.07	44±5	0.3±0.02
AITC (1 nmol/site)	$10\pm4^{***}$	$0.6\pm0.07^{*}$	$9\pm2^{***}$	$1.7\pm0.2^{***}$
AITC (1 nmol/site) + HC-030031 (300 nmol/site)	$51\pm5^{\#}$	$0.3\pm0.1^{\#}$	$36\pm9^{\#}$	$0.9\pm0.3^{\#}$
AITC (1 nmol/site) + Camphor (150 nmol/site)	$45\pm6^{\#}$	$0.3\pm0.3^{\#}$	$46\pm7^{\#}$	$0.9\pm0.2^{\#}$

The data are presented as the means ± SEM. Mechanical hyperalgesia was expressed as 50% mechanical paw withdraw threshold (PWT in g). $^{*}P < 0.05$, $^{***}P < 0.001$ compared with vehicle; $^{\#}P < 0.05$, $^{\#\#\#}P < 0.001$ compared with the AITC treated group; two-way ANOVA followed by Bonferroni's post hoc test.

MSU i.a. injection increases TRPA1 expression in synovial tissue and CGRP release

MSU increased TRPA1 expression 2.5-fold in synovial tissue 4 hours, but not 1 hour, after i.a. injection of MSU (Fig. 3A). TRPA1 neuronal activation was assessed by measuring CGRP-LI release by MSU, which produced a 1.5-fold increase in synovial lavage fluid compared to vehicle, HC-030031 administration reduced CGRP-LI release (Fig. 3B). The H_2O_2-detoxifying enzyme catalase abolished CGRP-LI release after MSU injection, suggesting a role of endogenous H_2O_2 in the TRPA1-mediated MSU response (Fig. 3B).

MSU challenge increased H_2O_2 production in synovial tissue

MSU injection (25 mg/mL) at 1 and 4 hours increased synovial tissue H_2O_2 levels (Fig. 3C). The MSU challenge also increased synovial membrane H_2O_2 production at different time points (0.25 to 4 hours). In addition, pretreatment of the synovial membranes with colchicine (10 µM for 1 hour) reduced the H_2O_2 production (Fig. 3D).

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

H_2O_2 (3 µmol/site, i.a.) injection induced mechanical allodynia and edema. TRPA1 antagonists (HC-030031 and camphor) reduced the mechanical allodynia and i.a. edema formation induced by i.a. injection of H_2O_2 after 1 and 4 hours (Fig. 4A-B). Catalase (300 UI/site) diminished either MSU- or H_2O_2-induced allodynic and edematogenic responses at 1 and 4 hours (Fig. 4C-D). The reducing agent DTT (20 nmol/site), which reverses TRPA1 activation by H_2O_2 in vitro,[31] also decreased MSU- or H_2O_2-induced allodynia and edema (Fig. 4E-F).

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

We found that MSU (1.25 mg/site, i.a.) significantly increased the number of emigrated neutrophils in H&E-stained slices of articular tissue at 4 hours, but not 1 hour, after injection (Fig. 5A-B). The inflammatory effect induced by MSU was reduced by co-injection (300 nmol/site, i.a.) or oral pretreatment with HC-030031 (300 µmol/kg, 1 hour before) (Fig. 5A-B). Finally, HC-030031 or catalase injection reduced the number of neutrophils (Fig. 5C), the MPO activity (Fig. 5D), the plasma

extravasation (Fig. 5E), and the increase in IL-1β content (Fig. 5F) observed in the synovial tissue 4 hours after i.a. MSU challenge.

DISCUSSION

Hyperalgesia and edema are relevant symptoms observed in patients affected by acute gout attacks, and the reduction of pain hypersensitivity and inflammation states is a main therapeutic goal for this condition.[32] The major finding of the present study is that pharmacological blockade or genetic ablation of the TRPA1 channel markedly decreases the mechanical hyperalgesia and the entire inflammatory repertoire produced by i.a. injection of MSU, which represents a predictive rodent model of acute gout attacks. Accordingly, different studies have described the contribution of TRPA1 in hyperalgesia and edema in inflammatory pain models.[10-12]

TRPA1 is co-expressed with TRPV1 peptidergic sensory neurons.[33] Thus, the known ability of capsaicin to produce desensitization of TRPV1 expressing neurons, results in profound desensitization of TRPA1-peptidergic sensory afferents.[34] In addition, the use of capsaicin as a counter-irritant to treat gout pain is probably based on the desensitization properties of the drug.[35] Here, we showed that capsaicin desensitization causing defunctionalization of TRPA1-expressing neurons produced antinociceptive and anti-inflammatory effects. The observation that capsaicin treatment decreased TRPA1 immunoreactivity in the synovial tissue, may be explained by the ablation of sensory nerve terminals associated with the desensitizing effect of this TRPV1 agonist.

MSU crystals or uric acid does not directly stimulate the TRPA1, in contrast, hydrogen peroxide promotes TRPA1 activation.[9, 13] Injection of MSU (i.a.) increased the hydrogen peroxide levels in synovial membranes in vitro, an effect reduced by colchicine, indicating a role of MSU phagocytosis in this process. Accordingly, MSU challenge induced production of oxidative substance from resident cells and neutrophil, and both responses were reduced by colchicine.[30, 36, 37] Thus, by analogy, we propose that resident synovial cells, which have the potential to phagocytosis MSU crystals, generate hydrogen peroxide when challenged with MSU. In the normal synovial tissue, type A synoviocytes are known to exert a macrophage-like behavior, with the associated phagocytic activity.[38, 39] Consistent with these findings, i.a. injection of hydrogen peroxide evoked nociception and inflammation in a

TRPA1-dependent manner. In addition, catalase or the reducing agent DTT decreased these effects in rats. Accordingly, gout patients show levels of oxidative stress byproducts that are higher than those present in normal subjects.[1, 5]

MSU-mediated inflammation has been linked to IL-1β production following activation of infiltrating cells.[40-42] Indeed, neutralization of IL-1β has been explored as a new strategy for the relief of gout pain.[43, 44] However, present data indicate that TRPA1 activation, presumably by H_2O_2, is upstream to neutrophil infiltration and increased IL-1β production. In fact, early blockade of TRPA1 receptor or of H_2O_2 production by catalase reduced the effects of MSU on IL-1β, MPO activity or neutrophil accumulation in the synovial space.

While the delayed increase is consistent with the time usually required either for new protein generation or for transport to the nerve terminals, the present findings suggest that increased TRPA1 expression is more likely linked to the maintenance of the hypersensitivity and inflammatory condition evoked by MSU rather than to its onset. It is possible, as observed by others,[45] that released inflammatory mediators such as IL-1β favor TRPA1 expression and contribute, by this and other mechanisms, to increased firing of nociceptive neurons, leading to nociception.[12, 46]

In conclusion, as previously observed in subcutaneous tissue, our present data support the notion that early TRPA1 activation by oxidative substances in the synovial tissue mediated nociceptive and inflammatory responses induced by MSU i.a. injection. These findings, pointed to TRPA1 antagonism as a possible novel therapeutic option for gout treatment.

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FIGURES AND LEGENDS

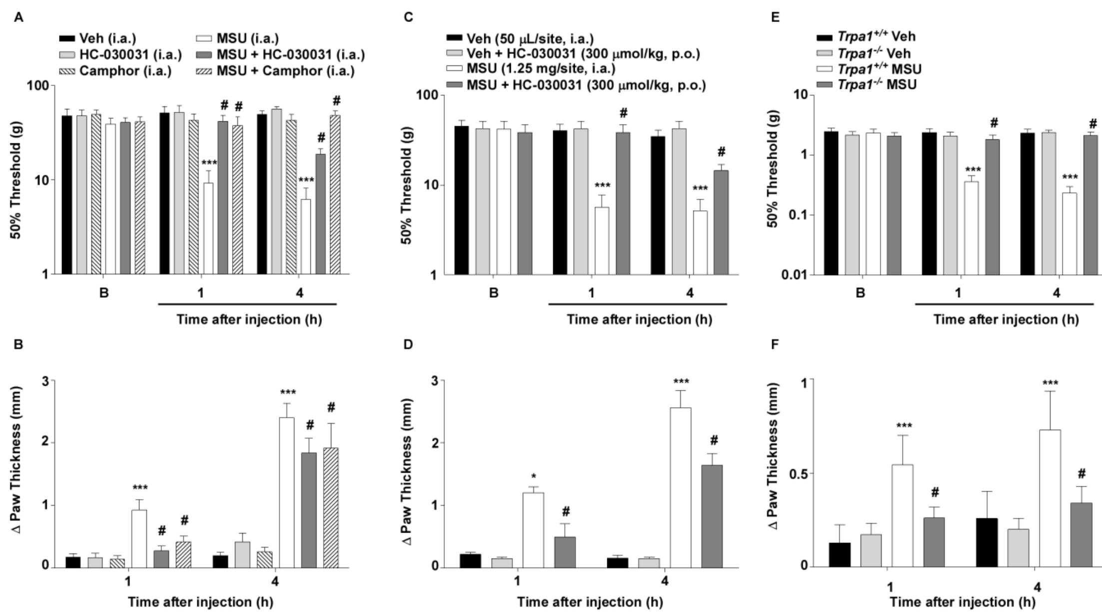

Fig. 1. *Inflammatory responses induced by intra-articular (i.a.) injection of monosodium urate crystals were mediated by TRPA1 channel activation in rodents.* The (A) mechanical allodynia and (B) edema caused by i.a. injection of MSU were reduced by co-injection with the selective and poorly selective TRPA1 antagonists HC-030031 (300 nmol/site, i.a.) and camphor (150 nmol/site, i.a.), respectively. HC-030031 oral pretreatment (300 µmol/kg, 1 hour before MSU injection) also reduced the (C) mechanical allodynia and (D) edema induced by i.a. MSU injection. TRPA1 deficient mice (*Trpa1*$^{-/-}$) showed reduced (E) mechanical allodynia and (F) edema formation to i.a. MSU injection compared with *Trpa1*$^{+/+}$ mice. The baseline threshold of animals was represented as B in the graphs. Each column represents the mean ± S.E.M. of six rats or seven mice. The asterisks denote the significance levels. *P < 0.05, ***P < 0.001 compared with the vehicle (Veh)-treated group (*Trpa1*$^{+/+}$ treated mice in E and F); or $^{\#}$P < 0.05 compared with the MSU-treated group (*Trpa1*$^{+/+}$ treated mice in E and F); two-way ANOVA followed by Bonferroni's post hoc test.

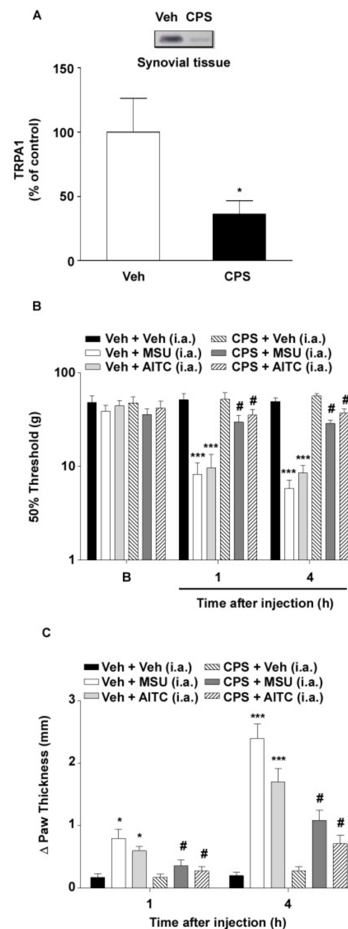

Fig. 2. *Ablation of TRPA1-positive fibers largely reduced inflammatory responses elicited by injection of monosodium urate crystals.* (A) Western blot (inset) showing TRPA1 immunoreactivity in synovial tissue samples 7 days after the injection of capsaicin (CPS, 2%) or vehicle (Veh). Western blot results are expressed as % of control. The perineural injection of CPS (2%) seven days before the intra-articular (i.a.) injection of monosodium urate (MSU, 1.25 mg/site) crystals, the TRPA1 agonist AITC (1 nmol/site), or vehicle reduced the (B) mechanical allodynia and (C) edema formation induced by MSU or AITC in rats. The baseline threshold of animals was represented as B in the graphs. Each column represents the mean ± S.E.M. of six rats, except for western blot when three to four samples were used. The asterisks denote the significance levels. *$P < 0.05$, ***$P < 0.001$ compared with the Veh-treated group (pretreated with Veh); $^{\#}P < 0.05$ compared with the MSU- or AITC-treated group (pretreated with Veh); Student's t test (in A) or two-way ANOVA followed by Bonferroni’s post hoc test (in B-C).

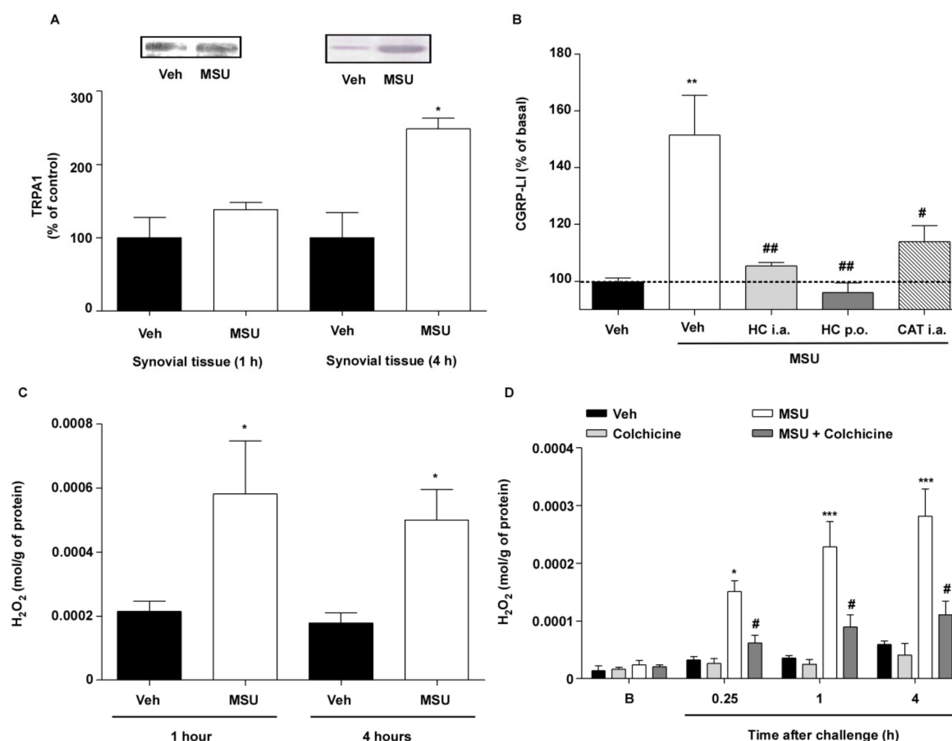

Fig. 3. *Monosodium urate crystal-elicited nociceptive and edematogenic responses were accompanied by an increase in TRPA1 expression, release of calcitonin gene-related peptide (CGRP), and H_2O_2 production by the synovial tissue.* (A) Western blot (inset) showing TRPA1 immunoreactivity in synovial tissue at 1 and 4 hours after the i.a. injection of MSU (1.25 mg/site) or vehicle (Veh). Western blot results are expressed % of control. (B) Calcitonin gene-related peptide-like immunoreactivity (CGRP-LI) was increased 15 minutes after MSU (1.25 mg/site) i.a. injection, an effect reduced by co-injection with HC (300 nmol/site) or CAT (300 UI/site), or by oral pretreatment with HC (300 µmol/kg, 1 hour). The CGRP-LI assay was performed in the synovial lavage samples obtained from pretreated rats. (C) The intra-articular (i.a.) injection of monosodium urate (MSU, 1.25 mg/site) crystals increased H_2O_2 content in synovial tissue 1 or 4 hours after administration compared with samples from vehicle (Veh, 50 µL/site)-injected animals. (D) Incubation with MSU (25 mg/mL) of rat synovial membranes *in vitro* enhances the production of H_2O_2 at different time points (0.25 to 4 hours), and pretreatment of the membranes with colchicine (10 µM for 1 hour) reduced the H_2O_2 production by rat synovial membranes challenged with MSU (25 mg/mL). The basal level of H_2O_2 production was represented as B in D. The data are expressed as the mean ± S.E.M. of six rats or six samples, except for western blot when three to four samples were used. The asterisks denote the significance levels. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared with the Veh-treated group; or $^{\#}P < 0.05$, $^{\#\#}P < 0.05$ compared with the MSU-treated group; Student's t test (in A), and one-way ANOVA (in B) or two-way ANOVA followed by Bonferroni's post hoc test (in C-D).

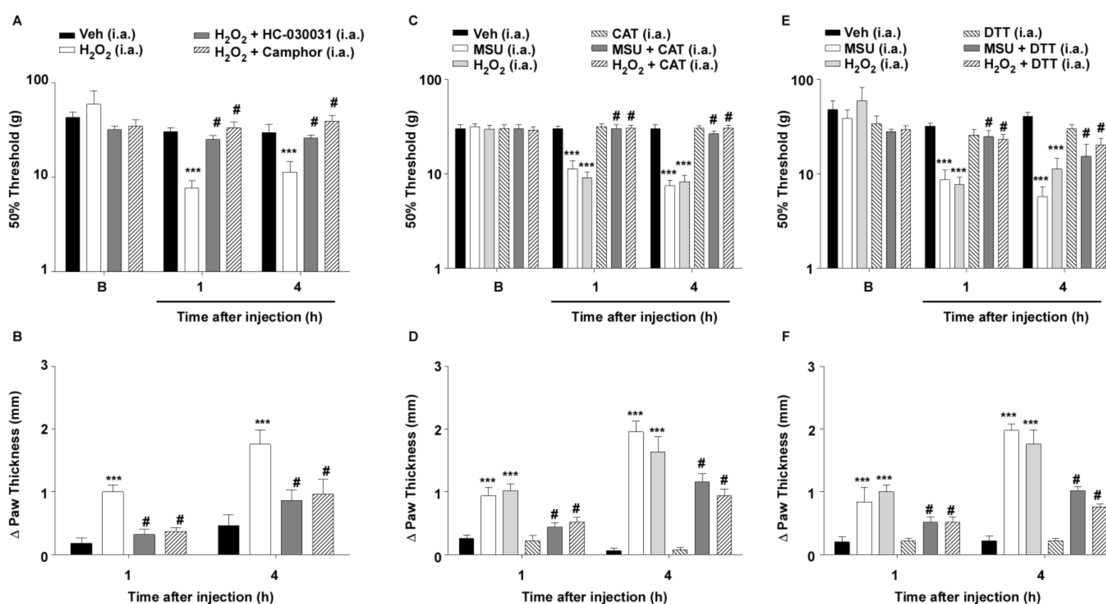

Fig. 4. *Monosodium urate crystal-induced nociception and edema were mediated via activation of TRPA1 receptors by hydrogen peroxide.* The selective and nonselective TRPA1 antagonists HC-030031 (300 nmol/site, i.a.) and camphor (150 nmol/site, i.a.), respectively, when co-injected with H_2O_2 (3 μmol/site, i.a.) decreased the (A) mechanical allodynia and (B) edema formation induced by this TRPA1 agonist. The co-injection of catalase (CAT, 300 UI/site, i.a.) with H_2O_2 (3 μmol/site, i.a.) or monosodium urate (MSU, 1.25 mg/site, i.a.) crystals reduced the (C) mechanical allodynia and (D) edema formation. The reducing agent dithiothreitol (DTT, 20 nmol/site, i.a.) was co-injected with MSU (1.25 mg/site, i.a.) or H_2O_2 (3 μmol/site, i.a.), and it reduced the (E) mechanical allodynia and (F) edema induced by i.a. injection of MSU or H_2O_2. The baseline threshold of animals was represented as B in the graphs. Each column represents the mean ± S.E.M. of six rats. The asterisks denote the significance levels. ***$P < 0.001$ compared with vehicle (Veh)-treated group; or $^{\#}P < 0.05$ in comparison to MSU or H_2O_2 treated group; two-way ANOVA followed by Bonferroni's post hoc test.

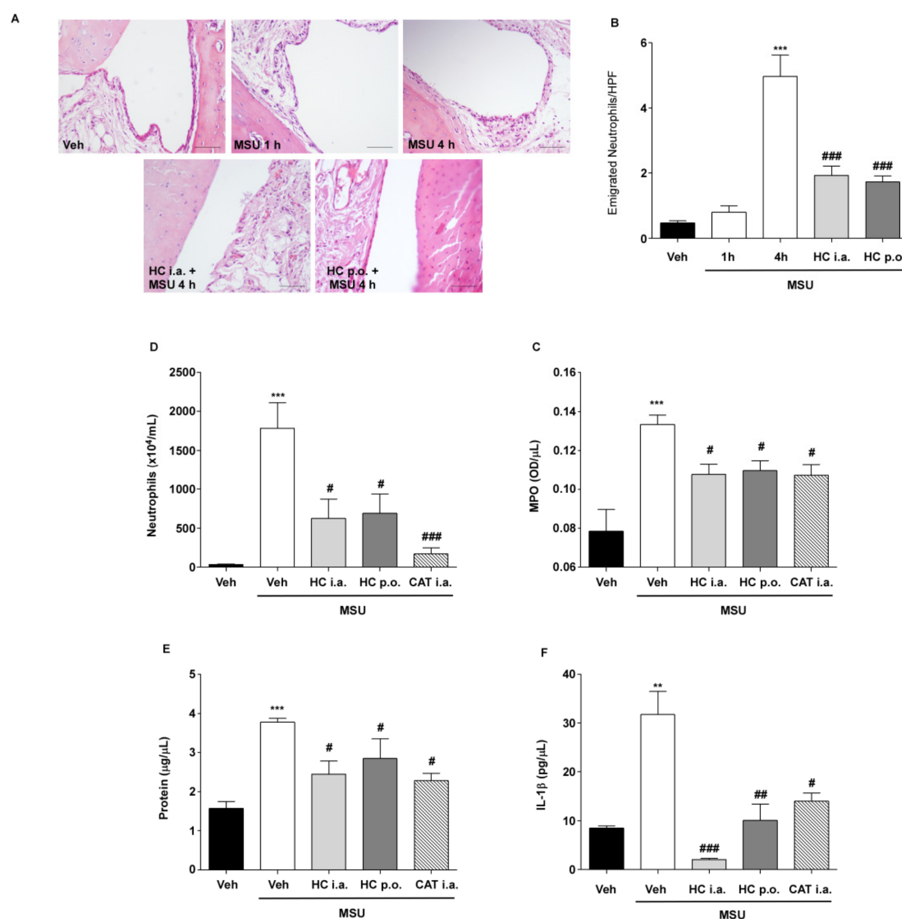

Fig. 5. *Monosodium urate crystal-induced inflammatory response is largely reduced by TRPA1 antagonism and blockade of H_2O_2 production.* (A) Representative images and (B) pooled data showing the accumulation of emigrated neutrophils induced by the i.a. injection of MSU (1.25 mg/site) in rats at different time points (1 and 4 hours), evaluated by H&E staining and histological analysis. The i.a. co-injection of MSU with the TRPA1 antagonist HC-030031 (HC, 300 nmol/site) and oral (p.o.) pretreatment with HC (300 µg/kg, 1 hour) significantly reduced the emigration of neutrophils 4 hours post MSU injection. (HPF, high power fields, X200, scale bar 100 µm). The i.a. co-injection of MSU (1.25 mg/site) with HC (300 nmol/site) or catalase (CAT, 300 UI/site) and oral pretreatment with HC-030031 (300 µg/kg, p.o., 1 hour) decreased the (C) neutrophil infiltration, (D) MPO activity, (E) plasma extravasation, and (F) interleukin-1β (IL-1β) levels observed 4 hours after i.a. MSU injection. All the measurements were performed using synovial lavage samples obtained from pretreated rats. Each column represents the mean ± S.E.M. of six samples. The asterisks denote the significance levels. ***P < 0.001 compared with the vehicle (Veh)-treated group; or #P < 0.05, ##P < 0.01, ###P < 0.001 compared with the MSU-injected group; one-way ANOVA followed by Bonferroni's post hoc test.

DISCUSSÃO

A gota é uma forma comum de artrite inflamatória, que apresenta um impacto considerável na qualidade de vida dos pacientes (RICHETTE; BARDIN, 2010; TERKELTAUB, 2010). Porém mesmo sendo uma doença descrita deste a antiguidade e com uma grande quantidade de estudos realizados até o momento, os mecanismos que estão envolvidos na dor e inflamação observadas no ataque agudo de gota ainda não foram totalmente esclarecidos. Assim o tratamento adequado do ataque agudo de gota ainda apresenta limitações tanto pelos efeitos adversos associados quanto pela reduzida eficácia dos fármacos disponíveis (MALAWISTA; DE BOISFLEURY; NACCACHE, 2011; TERKELTAUB, 2010). Neste estudo, podemos observar através de dados bioquímicos, farmacológicos e genéticos que o receptor TRPA1 e a produção de H_2O_2 apresentam um papel relevante na nocicepção e também na inflamação observada em modelos de ataque agudo de gota.

Inicialmente, observamos o envolvimento do receptor TRPA1 em dois modelos de nocicepção e inflamação associados à administração intraplantar ou intra-articular de cristais de MSU em roedores. Quando este estímulo foi administrado por via intraplantar observamos o desenvolvimento de nocicepção espontânea, edema e também alodínia ao frio. Porém, como a administração intra-articular de cristais de MSU é um modelo mais fidedigno para o estudo da participação de mecanismos envolvidos no ataque agudo de gota (HOFFMEISTER et al., 2011), também avaliamos a participação do canal TRPA1 após administração i.a. de cristais de MSU em roedores. Além disso, a administração intra-articular de cristais de MSU em roedores induziu o desenvolvimento de hiperalgesia mecânica e também edema articular. É importante observar que o desenvolvimento de hiperalgesia e edema articular são sintomas clínicos importantes observados em ataques agudo de gota em pacientes, e então a redução destes sintomas seria um fator importante para o tratamento desta patologia dolorosa (DALBETH; LINDSAY, 2012). Também, anteriormente, já foi indicado que a administração de cristais de MSU por via intra-articular seria um modelo relevante para o delineamento de novos analgésicos em modelos pré-clínicos (CODERRE; WALL, 1987,1988), e estas

respostas poderiam estar associadas à ativação de fibras sensoriais TRPV1 positivas (CODERRE; WALL, 1987, 1988; OTSUKI et al., 1986). Ainda, a capsaicina um agonista TRPV1 já mostrou ação analgésica em diferentes formas de artrite (DEAL et al., 1991) e também pode reduzir a dor observada na gota (WALKER; MCCLEANE, 2002).

O ataque agudo de gota é uma situação dolorosa onde podemos observar o desenvolvimento de dor em queimação e também alodínia ao frio (DORWART, 2004; LINDSAY et al., 2011), mostrando que termonociceptores encontrados em neurônios sensoriais poderiam estar envolvidos nesta patologia. Dessa maneira, recentemente nosso grupo de estudo mostrou a participação do receptor TRPV1 na nocicepção e no edema induzidos pela administração de cristais de MSU em roedores (HOFFMEISTER et al., 2011). O receptor TRPA1 é expresso conjuntamente com o receptor TRPV1 em aproximadamente 30% das fibras sensoriais peptidérgicas TRPV1-positivas (STORY et al., 2003), e o tratamento com agonistas TRPV1 normalmente pode desensibilizar e reduzir a expressão também do receptor TRPA1 em neurônios sensoriais peptidérgicos (PECZE et al., 2009). Além disso, as fibras sensoriais que expressam o receptor TRPA1 contem neuropeptídeos como a SP e o CGRP, que quando liberados pela ativação deste receptor levam ao desenvolvimento de respostas associadas à inflamação neurogênica.

Previamente, notamos que a desensibilização das fibras sensoriais TRPV1-positivas pelo tratamento com capsaicina por via perineural poderia levar à redução das respostas associadas à administração dos cristais de MSU (HOFFMEISTER et al., 2011). Neste estudo, observamos que este tratamento poderia reduzir a expressão do receptor TRPA1, em amostras de tecido sinovial ou ainda de nervo ciático, e também leva a diminuição da nocicepção e inflamação associadas ao tratamento com cristais de MSU em roedores por via intraplantar ou ainda intra-articular. Mesmo que inicialmente o receptor TRPA1 tenha sido indicado como um sensor à baixas temperaturas, atualmente tem sido proposto que este canal seria associado principalmente ao desenvolvimento de alodínia ao frio em modelos de dor inflamatória ou neuropática (DA COSTA et al., 2010; DEL CAMINO et al., 2010; NASSINI et al., 2011). Neste estudo, também demonstramos que o TRPA1 poderia estar associado à alodínia ao frio após a administração intraplantar de cristais de MSU, porém o receptor TRPV1 não estaria envolvido nestas respostas. Também,

observamos que a administração de um agonista TRPA1, o AITC poderia induzir respostas nociceptivas e edematogênicas similares à administração intraplantar ou intra-articular de cristais de MSU, mostrando que a ativação deste canal nestes tecidos é relevante para o desenvolvimento de dor e inflamação.

Recentemente, o receptor TRPA1 tem sido indicado como um alvo importante para o desenvolvimento de dor em modelos pré-clinicos de dor neuropática e inflamatória em roedores (ANDRADE; MEOTTI. CALIXTO, 2012; BARALDI et al., 2010; BAUTISTA; PELLEGRINO; TSUNOZAKI, 2013). No presente estudo, o objetivo principal foi observar se este sensor poderia também estar envolvido na dor e inflamação observada em modelos de gota. Utilizando diferentes estratégias farmacológicas podemos avaliar que a administração local (intraplantar ou intra-articular) ou ainda o tratamento por via oral com antagonistas TRPA1 reduziram as respostas nociceptivas e edematogênicas associadas à injeção de cristais de MSU. Também, utilizando uma ferramenta genética podemos avaliar que animais que não expressam o receptor TRPA1 funcional também apresentaram respostas diminuídas após a administração de cristais de MSU por via intraplantar ou intra-articular. Assim, estes dados podem confirmar que o receptor TRPA1 tem um papel importante nestes modelos de dor associados ao ataque agudo de gota. Diferentes estudos têm descrito a importância do receptor TRPA1 no desenvolvimento de alodínia e edema em modelos de dor inflamatória (BONET et al., 2012; DA COSTA et al., 2010; DEL CAMINO et al., 2010; LENNERTZ et al., 2012; MOILANEN et al., 2012). Além disso, aqui demonstramos que um antagonista do receptor TRPA1 que é biodisponível quando administrado por via oral, o HC-030031 (MCNAMARA et al., 2007), pode apresentar efeito antinociceptivo e anti-inflamatório, sendo este um indicativo pré-clinico que o tratamento com este composto poderia ser uma estratégia interessante para o tratamento do ataque agudo de gota.

Os ataques agudos de gota são associados a uma resposta inflamatória dolorosa mediada pelos cristais de MSU e já foi observado que a produção de IL-1β após a ativação de células inflamatórias seria um fenômeno importante nestes mecanismos (MATSUKAWA et al., 1998; SCANU et al., 2010; TORRES et al., 2009). Observando que a liberação de IL-1β é um fator relevante nas respostas mediadas pelos cristais de MSU, a neutralização desta citocina tem sido explorada como um alvo para o tratamento da dor e inflamação relacionadas ao ataque agudo de gota (SCHLESINGER et al., 2012; SCHUMACHER et al., 2012). Tanto a infiltração de

leucócitos quanto o conteúdo de IL-1β foram avaliados nos tecidos plantar e articular após a administração de MSU. Interessantemente foi observado um aumento de ambas as respostas, as quais foram reduzidas pela administração do antagonista do receptor TRPA1. Estes resultados são de grande pertinência, uma vez que estes episódios são dois fenômenos associados ao ataque agudo de gota (TERKELTAUB, 2010; TORRES et al., 2009). Assim, a redução destes fenômenos inflamatórios por um antagonista TRPA1 é um fato relevante mostrando que a ativação deste receptor, poderia ser um mecanismo importante para o desenvolvimento dos ataques agudos de gota.

Também, foi observado um aumento da expressão dos receptores TRPV1 e TRPA1 em amostras de nervo ciático após 6 horas da administração intraplantar de cristais de MSU, e um aumento da expressão do receptor TRPA1 no tecido sinovial após 4 horas da administração intra-articular de cristais de MSU, mas não após 1 hora. Recentemente, foi demonstrado que a IL-1β seria capaz de aumentar a expressão do receptor TRPA1 em cultura de sinoviócitos (HATANO et al., 2012). A partir disso, podemos hipotetizar que o bloqueio inicial do receptor TRPA1 no tecido sinovial poderia reduzir a produção de mediadores inflamatórios associados que poderiam levar ao aumento da expressão do canal no tecido sinovial. Ainda, o aumento da expressão do receptor TRPA1 pode aumentar as descargas mediadas pelos neurônios nociceptivos em modelos de dor inflamatória (DA COSTA et al., 2010; LENNERTZ et al., 2012). Enquanto, a infiltração de leucócitos, a produção de IL-1β e o aumento da expressão do receptor TRPA1 após a administração intraplantar dos cristais de MSU estão relacionados à formação de edema (aumentado após 6 horas da administração i.pl. dos cristais de MSU), a nocicepção pode ser avaliada ainda em um período inicial e é mais intensa nestes períodos, a mesma relação foi observada para a injeção intra-articular dos cristais de MSU.

Além disso, observamos que a administração intraplantar ou intra-articular de MSU induziu respostas nociceptivas em períodos onde não foi observada a infiltração de neutrófilos no tecido sinovial ou a produção de IL-1β, ou ainda outros parâmetros inflamatórios após a administração intra-articular de cristais de MSU, e estas foram reduzidas pelo tratamento com um antagonista do receptor TRPA1. Assim, estes fenômenos iniciais poderiam ser provocados pela ativação do receptor TRPA1 por um agonista endógeno produzido após a administração dos cristais de MSU. De tal modo, o bloqueio desta ativação poderia bloquear este fenômeno inicial

associado ao ataque agudo de gota. Também, observamos que a ativação inicial do receptor TRPA1 após a administração dos cristais de MSU por via intra-articular poderia levar à liberação de peptídeos vasoativos, como o CGPR, favorecendo a infiltração celular, este fenômeno foi reduzido por um antagonista TRPA1 e pela catalase. De fato, o antagonismo inicial do receptor TRPA1 ou ainda o bloqueio da produção de H_2O_2 utilizando a catalase reduziu os conteúdos de IL-1β, a atividade da mieloperoxidase e o acúmulo de neutrófilos no espaço sinovial após a administração intra-articular de MSU.

Já foi demonstrado que os cristais de MSU ou o ácido úrico podem ativar diretamente diferentes tipos celulares, e em muitos casos esta ativação é independente da fagocitose dos cristais (FALASCA et al., 1993; NG et al., 2008; ROCK; KATAOKA; LAI, 2013). Porém, quando avaliamos se o ácido úrico ou os cristais de MSU poderiam levar a indução de influxo de cálcio diretamente em culturas de neurônios do GRD não observamos uma mobilização das respostas, mostrando que estes não podem ativar diretamente os neurônios sensoriais. Assim, como o ácido úrico ou os cristais não podem ativar diretamente o receptor TRPA1 expresso em neurônios sensoriais. Uma alternativa seria que o MSU estaria provocando a ativação deste canal por mecanismos indiretos, o que já poderia ser sugerido pela cinética de resposta após a injeção dos cristais de MSU por via intraplantar. Quando administrados por via intraplantar os cristais de MSU induzem uma resposta nociceptiva espontânea após 5-10 minutos da administração, enquanto que a administração de AITC ou capsaicina causa uma resposta intensa inicial após cerca de 1-2 minutos (TREVISAN et al., 2013a).

A estimulação de células residentes e também de células inflamatórias que infiltram os tecidos por cristais de MSU ou ácido úrico pode levar a produção de espécies reativas de oxigênio (ABRAMSON; HOFFSTEIN; WEISSMANN, 1982; FALASCA et al., 1993; THOMAS, 1992). Neste estudo, avaliamos que a administração de cristais de MSU por via intraplantar ou intra-articular além de induzir dor e edema também poderia levar ao aumento dos níveis de H_2O_2 nos tecidos injetados. Após a administração dos cristais de MSU por via intraplantar os níveis de H_2O_2 estavam elevados de 0.25 h até 6 horas após a administração, mas em níveis reduzidos. Então o conteúdo de H_2O_2 nos tecidos parece ser relevante para o desenvolvimento da nocicepção inicial, porém é um evento acessório para o aparecimento de edema, que pode envolver principalmente mediadores pró-

inflamatórios, efeitos similares foram observados para a administração intra-articular dos cristais de MSU. Previamente, o H_2O_2 foi identificado como um agonista endógeno do receptor TRPA1 (ANDERSSON et al., 2008; KEEBLE et al., 2009; SAWADA et al., 2008). Então, é possível que a exposição aos cristais de MSU leve a produção de H_2O_2 e então este mediador ative o receptor TRPA1 em neurônios sensoriais peptidérgicos, produzindo as respostas nociceptivas e inflamatórias associadas à administração de cristais de MSU. Mesmo que o receptor TRPA1 expresso em células neuronais pareça ter um papel predominante nas respostas associadas aos cristais de MSU, outras células que também expressam este canal, como as células endoteliais e sinoviais, poderiam também estar envolvidas nos efeitos mediados pelos cristais de MSU (ANDRADE; MEOTTI; CALIXTO, 2012).

Além disso, a exposição de membranas sinoviais de ratos *in vitro* aos cristais de MSU induz a produção de H_2O_2 e este fenômeno é reduzido pelo tratamento prévio do tecido sinovial com colchicina. Estes resultados estão de acordo com dados prévios mostrando que a produção de espécies reativas de oxigênio após o exposição de cristais de MSU à células residentes ou neutrófilos poderia ser reduzido pela colchicina (CHIA; GRAINGER; HARPER, 2008; GAUDRY et al., 1993; MARTIN et al., 2010; MARTIN; WALTON; HARPER, 2009). Estes dados levam a suposição que células sinoviais poderiam estar envolvidas na produção de H_2O_2 quando em contato com os cristais de MSU. Ainda, já que a colchicina é capaz de reduzir este fenômeno podemos hipotetizar que estas células seriam capazes de fagocitar os cristais de MSU. No tecido sinovial normal os sinoviócitos do tipo A se comportam como macrófagos e podem então ser capazes de fagocitar os cristais de MSU (MALAWISTA; DE BOISFLEURY; NACCACHE, 2011; SCHUMACHER; PHELPS; AGUDELO, 1974).

Similar aos agonistas do canal TRPA1, a injeção de H_2O_2 por via intraplantar ou intra-articular foi capaz de induzir nocicepção e edema, e estas respostas foram reduzidas pelos antagonistas TRPA1. Também as respostas mediadas pela administração de H_2O_2 foram observadas rapidamente após a administração diferente do que foi avaliado para a administração de cristais de MSU. Além disso, para avaliar a participação do H_2O_2 nas respostas induzidas pelos cristais de MSU, utilizamos o DTT um agente redutor permeável à célula, este é capaz de ligar-se aos resíduos de cisteína e então reverter a ativação deste receptor pelo H_2O_2 (ANDERSSON et al., 2008; TAKAHASHI et al., 2008). Aqui, este agente redutor foi

capaz de proteger os animais quanto à indução das respostas nociceptivas e edematogênicas causadas pela administração dos cristais de MSU. Ainda, efeitos similares foram avaliados para a administração da enzima catalase, mostrando que o bloqueio da produção de H_2O_2 é um fator relevante nos mecanismos envolvidos no ataque agudo de gota. De maneira interessante já foi descrito que pacientes com gota mostram um conteúdo aumentado de substâncias oxidativas em relação a indivíduos normais, sendo que este estado poderia corroborar para o desenvolvimento da dor e da inflamação observas na gota (AMARAL et al., 2012; TERKELTAUB, 2010). Além disso, a vitamina C, um potente antioxidante, foi descrita como um agente que causa a redução da incidência de gota (HUANG et al., 2005; JURASCHEK et al., 2011; SHEN; JI, 2011).

Assim, todos os dados obtidos neste estudo nos levam a concluir que a ativação inicial do receptor TRPA1 por substâncias oxidantes é capaz de mediar as respostas nociceptivas e inflamatórias produzidas após a injeção dos cristais de MSU. Dessa maneira, a partir dos resultados obtidos neste estudo propomos que a administração de antagonistas do receptor TRPA1 por via i.a. ou por via oral, além do uso de catalase por via i.a. poderiam ser exploradas como novas alternativas para o tratamento do ataque agudo de gota.

5 CONCLUSÕES

Tendo em vista os resultados obtidos no presente estudo, pode-se concluir que:

a) a administração intraplantar ou intra-articular dos cristais de MSU causa nocicepção, edema, respostas inflamatórias como aumento do conteúdo de IL-1β e infiltração de neutrófilos no tecido de ratos e camundongos e estes eventos foram reduzidos pela administração de antagonistas do receptor TRPA1 ou ainda, pela redução da funcionalidade do canal TRPA1 por modificação genética de camundongos;

b) após a administração intraplantar ou intra-articular dos cristais de MSU podemos observar um aumento da expressão do canal TRPA1 em períodos de tempo que temos a presença de edema e inflamação;

c) a dessensibilização das fibras sensórias peptidérgicas TRPV1 e TRPA1-positivas pelo tratamento perineural com capsaicina reduziu a expressão dos receptores TRPV1 e TRPA1 e também a nocicepção e o edema induzidos pela administração intraplantar ou intra-articular dos cristais de MSU;

d) o ácido úrico ou os cristais de MSU não ativam diretamente o receptor TRPA1, uma vês que estes não são capazes de mobilizar o aumento no influxo de cálcio por canais TRPA1 expresso em culturas de neurônios dos gânglios da raiz dorsal;

e) a administração de cristais de MSU por via intraplantar ou intra-articular causa um aumento dos níveis de H_2O_2 nos tecidos injetados, efeito similar foi observado quando o MSU foi colocado em contato com membranas sinoviais, e este efeito foi reduzido pelo tratamento com colchicina. A injeção de H_2O_2 por via intraplantar ou intra-articular induziu nocicepção e edema, e estas respostas foram reduzidas pelos antagonistas TRPA1. Além disso, a respostas induzidas pelos cristais de MSU por injeção intraplantar ou intra-articular foram reduzidas pelo agente redutor DTT e pela catalase, e a administração de catalase também reduziu diferentes parâmetros inflamatórios avaliados após a administração dos cristais de MSU por via intra-articular, similar aos efeitos observados para o antagonista do receptor TRPA1 (Figura 5).

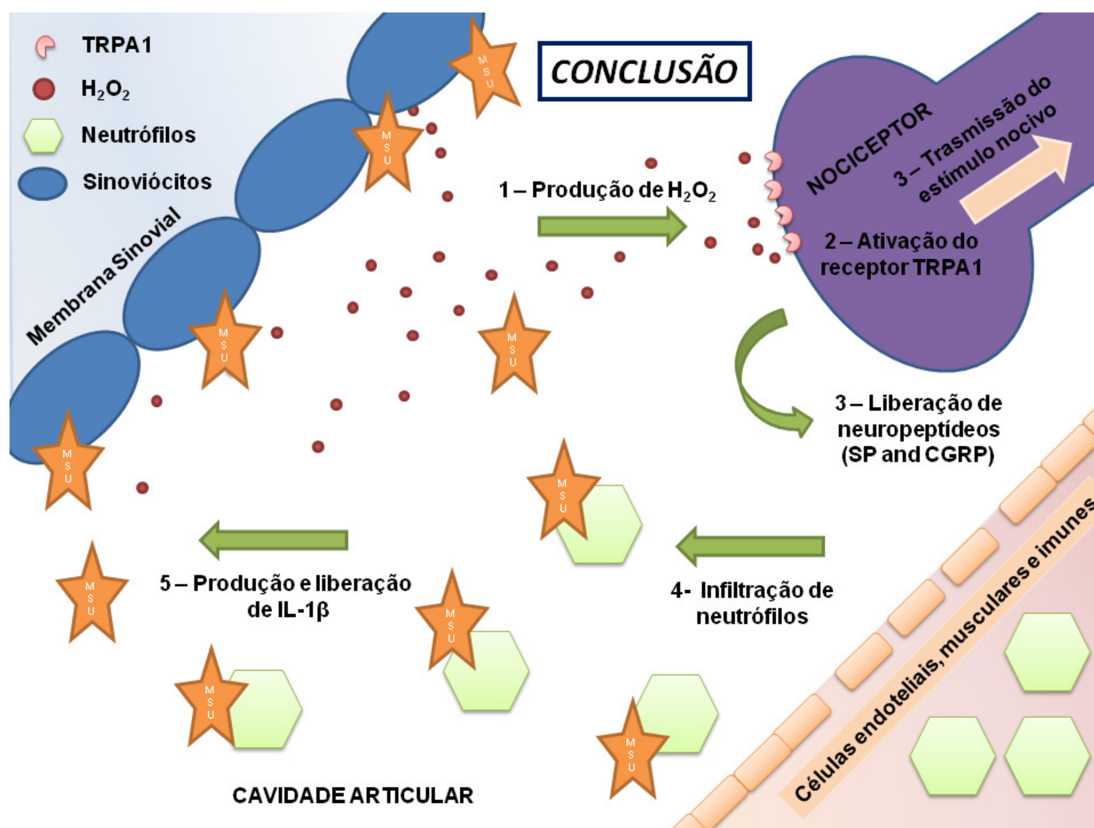

Figura 5 - Participação central do receptor TRPA1, mediada pela ativação por H_2O_2, nos mecanismos envolvidos na nocicepção e inflamação mediada pela injeção de cristais de MSU em roedores. (1) Primeiramente a fagocitose dos cristais de MSU pelos sinoviócitos (semelhantes à macrófagos do tipo A) poderia levar a produção de H_2O_2, (2) este levaria a ativação do receptor TRPA1 expresso em neurônios sensoriais nociceptivos, (3) causando a liberação de neuropeptídeos vasoativos como o CGRP e SP e também levando à produção de respostas dolorosas, (4) a estimulação de receptores para estes peptídeos expressos em células endoteliais, musculares e imunes poderia levar a infiltração de neutrófilos e então (5) a ativação do NALP3 e produção de IL-1β por estas células inflamatórias.

Todas estas evidências suportam a ideia que o receptor TRPA1 é um sensor relevante para o desenvolvimento de nocicepção e inflamação em um modelo de ataque agudo de gota em roedores. Então o bloqueio deste canal poderia ser explorado como um alvo farmacológico para o tratamento desta patologia.

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Apêndice A – Artigo publicado na revista Neuropharmacology: A novel, potent, oral active and safe antinociceptive pyrazole targeting kappa opioid receptors

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A novel, potent, oral active and safe antinociceptive pyrazole targeting kappa opioid receptors

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Pyrazole compounds are an intriguing class of compounds with potential analgesic activity; however, their mechanism of action remains unknown. Thus, the goal of this study was to explore the antinociceptive potential, safety and mechanism of action of novel 1-pyrazole methyl ester derivatives, which were designed by molecular simplification, using in vivo and in vitro methods in mice. First, tree 1-pyrazole methyl ester derivatives (DMPE, MPFE, and MPClE) were tested in the capsaicin test and all presented antinociceptive effect; however the MPClE (methyl 5-trichloromethyl-3-methyl-1*H*-pyrazole-1-carboxylate) was the most effective. Thus, we selected this compound to assess the effects and mechanisms in subsequent pain models. MPClE produced antinociception when administered by oral, intraperitoneal, intrathecal and intraplantar routes and was effective in the capsaicin and the acetic acid-induced nociception tests. Moreover, this compound reduced the hyperalgesia in diverse clinically-relevant pain models, including postoperative, inflammatory, and neuropathic nociception in mice. The antinociception produced by orally administered MPClE was mediated by κ-opioid receptors, since these effects were prevented by systemically pre-treatment with naloxone and the κ-opioid receptor antagonist nor-binaltorphimine. Moreover, MPClE prevented binding of the κ-opioid ligand [^{3}H]-CI-977 in vitro (IC_{50} of 0.68 (0.32–1.4) μM), but not the TRPV1 ([^{3}H]-resiniferatoxin) or the α_2-adrenoreceptor ([^{3}H]-idazoxan) binding. Regarding the drug-induced side effects, oral administration of MPClE did not produce sedation, constipation or motor impairment at its active dose. In addition, MPClE was readily absorbed after oral administration. Taken together, these results demonstrate that MPClE is a novel, potent, orally active and safe analgesic drug that targets κ-opioid receptors.

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Abbreviations: ClogP, coefficient of partition octanol/water; [^{3}H]-IDZ, [^{3}H]-idazoxan; MPCA, 5-hydroxy-3-methyl-5-(trichloromethyl)-1*H*-pyrazole-1-carboxamide; MPF4, methyl 5-hydroxy-4-methyl-5-(trifluoromethyl)-4,5-dihydro-1*H*-pyrazole-1-carboxylate; DMPE, methyl 3,5-dimethyl-1*H*-pyrazole-1-carboxylate; MPClE, methyl 3-methyl-5-(trichloromethyl)-1*H*-pyrazole-1-carboxylate; MPFE, Methyl 3-methyl-5-(trifluoromethyl)-1*H*-pyrazole-1-carboxylate; [^{3}H]-CI-977, [^{3}H]-(5*R*)-(5a,7a,8b)-(–)-*N*-methyl-*N*-(7-[1-pyrrolidinyl]-1-oxaspiro[4,5]dec-8-yl)-4-benzofuranacetamide hydrochloride; nor-BNI, nor-binaltorphimine; [^{3}H]-RTX, [^{3}H]-resiniferatoxin.

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pellet was discarded, and the supernatant was further centrifuged for 30 min at 35 000 × g at 4 °C. Binding assays were performed in duplicate in 500 μL of assay buffer supplemented with 0.25 mg/mL bovine serum albumin (to stabilise ligands in aqueous solution), membranes (0.5 mg/mL of protein), and [^{3}H]-RTX (2 nM) in the presence or absence of MPCIE (1–100 μM), capsaicin (10 μM) (positive control), or vehicle (0.01% dimethyl sulfoxide). To measure non-specific binding, 100 μM of non-radioactive RTX was included. The reaction was initiated by incubating the tubes at 37 °C for 60 min and stopped by transferring the tubes to ice bath and adding 100 μg of bovine α_1-acid glycoprotein. Finally, [^{3}H]-RTX-bound and unbound membranes were separated by centrifugation at 35 000 × g for 30 min at 4 °C. Radioactivity in the pellet was quantified by scintillation counting.

2.10. Evaluation of MPCIE antinociceptive effect in clinically-relevant pain models

The nociception was measured by the intensity of mechanical hyperalgesia, a painful hypersensitivity produced by previously innocuous mechanical stimuli, which is characteristic of pathological chronic pain. Mechanical hyperalgesia was assessed using von Frey filaments as described previously (Milano et al., 2008b). Mice were placed in clear plastic chambers (7 × 9 × 11 cm) on an elevated surface and allowed to acclimatize to their environment for 1 h before testing. The withdrawal response to von Frey filament (0.09 g, 10 times) was observed as described before (Milano et al., 2008b). The filament was applied to the mice hind paw plantar surface with a pressure causing the filament to bend. The frequency of mechanical paw withdrawal was determined before (baseline) and after nociception induction.

The preemptive effects of the orally administered drugs were evaluated in mice receiving a single administration of MPCIE (6 μmol/kg, p.o.) or vehicle (10 mg/mL, p.o.) 0.5 h before of the incisional procedure in the postoperative pain model. To evaluate the curative effect of the drugs, the animals received MPCIE (6 μmol/kg, p.o.) or vehicle (10 mg/mL, p.o.) 1 h after the incisional procedure for postoperative pain model, and 48 h or 7 days, after the procedure for the inflammatory or neuropathic pain model, respectively. Both in preemptive and curative treatments the mechanical hyperalgesia was recorded from 0.5 up to 6 h after administration.

2.10.1. Postoperative pain model: surgical incision-induced nociception

The postoperative pain model was carried out according to the procedure described to mice (Oliveira et al., 2013). Before surgery, baseline measurement of mechanical paw withdrawal was carried out. Later, mice were anesthetized with 2% halothane via a nose cone. After antiseptic preparation of the right hind paw, a 5-mm longitudinal incision was made with a number 11 blade through the skin and fascia of the plantar foot. The incision was started 2 mm from the proximal edge of the heel and extended towards the toes. The underlying muscle was elevated with a curved forceps, leaving the muscle origin and insertion intact. The skin was opposed with a single mattress suture of 6.0 nylon. Control mice underwent a sham procedure that consisted of anaesthesia and antiseptic preparation without an incision.

2.10.2. Inflammatory pain model: complete Freund's Adjuvant-induced nociception

The investigation of antinociceptive property of MPCIE was evaluated in the CFA (suspension of heat-killed *Mycobacterium tuberculosis* in oil, 1 mg/mL)-induced paw inflammation, a model of chronic inflammatory pain. Before CFA injection, baseline measurement of mechanical paw withdrawal (described above) was carried out. After baseline measurements, animals were anesthetized with halothane and 20 μL of CFA was injected subcutaneously in the plantar surface of the right hind paw of the animal (Rossato et al., 2011). Forty eight hours after, the development of mechanical hyperalgesia (reduction in the paw withdraw threshold) was assessed as previously described (Rossato et al., 2011). Control animals were i.pl. injected with PBS.

2.10.3. Neuropathic pain model: partial sciatic nerve ligation-induced nociception

Neuropathy was induced by a partial ligation of the sciatic nerve under deep anaesthesia (Trevisan et al., 2009). Briefly, mice were anesthetized intraperitoneally using a mixture of 90 mg/kg of ketamine plus 3 mg/kg of xylazine. A partial ligation of the right sciatic nerve was made by tying one-third to one half of the dorsal portion of the sciatic nerve. In sham-operated mice, the nerve was exposed without ligation.

2.11. Open-field and rotarod test

The effects of MPCIE and vehicle on forced or spontaneous locomotor activity were assessed using the rotarod test and the open-field test, respectively, as previously reported (Rossato et al., 2011). For the rotarod test, the animals were trained on the rotarod (3.7 cm in diameter, 8 rpm) until they could remain on the apparatus for 60 s without falling. On the day of the experiment, the animals were injected with MPCIE (6 μmol/kg, p.o.) or vehicle and subjected to the rotarod test 1 h later. The number of falls and latency to first fall from the apparatus were recorded for duration of 240 s. In the open-field test, the open field apparatus consisted of a box measuring (28 × 18 × 12 cm) with a floor that was divided into 15 identical areas. The animals were treated with MPCIE (6 μmol/kg, p.o.) or vehicle. One hour later, the animals were transferred to the apparatus and observed for 5 min. The number of areas crossed with all paws (crossings) and the number of rearing responses were recorded.

2.12. Platform sedation test

Sedation was measured by the latency of a mouse to completely step off a slightly raised platform, as described previously (Horan et al., 1991). Prior to treatment, the mice were tested once to establish baseline latency. Mice with a baseline latency under 15 s were not used. The mice received an oral dose of vehicle, MPCIE (6–600 μmol/kg). Thirty minutes later, the latency to step off the platform was tested. A 30-s cut-off was used. The per cent sedation was calculated by the following equation: [(test latency − baseline latency)/(30 s − baseline latency)] × 100. In addition, we calculated the peripheral restriction index, an indicator of the central nervous system liability of the compound (MPCIE) (Kumar et al., 2005), as the ratio of platform sedation ED_{50} and antinociception ED_{50} (sedation ED_{50}/antinociception ED_{50}).

2.13. Gastrointestinal transit

To evaluate whether MPCIE causes constipation, gastrointestinal transit was analysed, as described previously (Milano et al., 2008a). The mice were housed in cages without food for 18 h. Then, the mice were treated with MPCIE (6, 60, or 600 μmol/kg, p.o.), morphine (13 μmol/kg, p.o., positive control) or vehicle (10 mL/kg, p.o.). We have also administered the MPCIE (6 μmol/kg, p.o.) or vehicle (10 mL/kg, p.o.) for 7 days to observe the effect of MPCIE repeated administration on gastrointestinal transit. Forty minutes later of the treatment, the mice were given a standard charcoal meal (5% charcoal and 20% Arabic gum, 0.3 mL) by gavage. Twenty minutes after administration of the charcoal meal, the animals were euthanized, and their stomachs and small intestines were removed. The length of the intestine (from the pyloric sphincter to the ileo-caecal junctions) and the distance travelled by the charcoal meal were measured. Propulsive activity of the gut was evaluated by determining the percentage of gastrointestinally travelled charcoal, using the following equation: travelled (%) = 100 × (distance travelled/total gut length).

2.14. Quantification of MPCIE concentration by HPLC-DAD

All chemical were of analytical grade. Methanol and phosphoric acid purchased from Merck (Darmstadt, Germany). High performance liquid chromatography (HPLC-DAD) was performed with the HPLC system (Shimadzu, Kyoto, Japan), Prominence Auto Sampler (SIL-20A), equipped with Shimadzu LC-20AT reciprocating pumps connected to the degasser DGU 20A5 with integrator CBM 20A, UV–VIS detector DAD (photodiode) SPD-M20A and Software LC solution 1.22 SP1. Reverse phase chromatographic analyses were carried out under isocratic conditions using C18 column (4.6 mm × 250 mm) packed with 5 μm diameter particles; the mobile phase was acetonitrile: water (50:50, v/v) (Grillo et al., 2009). To quantify the presence of MPCIE in different tissues, animals were administered with MPCIE (6 μmol/kg, p.o.) and sacrificed 1 h latter to assess its disponibility in spinal cord, total brain, and plasma. Spinal cord (about 70 mg of tissue) and total brain (about 280 mg of tissue) samples were collect, homogenized in 1000 μL of PBS, and centrifuged at 5000 × g during 10 min, at 4 °C. Then, acetone (2:1) was added to the supernatant or plasma for deproteinization, followed of centrifugation at 10 000 × g during 10 min at 4 °C. The chromatography peak was confirmed by comparing its retention time with this of reference standard (MPCIE) and by DAD spectra (200–400 nm). The flow rate was 0.5 mL/min; injection volume 50 μL and the wavelength was 280 nm. All the samples and mobile phase were filtered through 0.45 μm membrane filter (Millipore) and then degassed by ultrasonic bath prior to use. A stock solution of MPCIE was prepared in the HPLC mobile phase at a concentration range of 0.0001–1 mM. Calibration curve for MPCIE was: $Y = 5905.6X + 31\ 062.1$ ($r = 0.9957$). All chromatography operations were carried out at ambient temperature and in triplicate.

2.15. Statistical analysis

The results are expressed as the mean ± S.E.M, with the exception of the ED_{50} (the dose of compound that produces 50% of effect relative to the control value) and IC_{50} (the concentration of compound that inhibited binding by 50% relative to the control value) values, which were reported as geometric means accompanied by respective 95% confidence limits. The ED_{50} and IC_{50} values were determined by a non-linear regression analysis using a sigmoid dose–response equation using GraphPad Software 5.0 software (GraphPad, USA). The maximum effect (E_{max}) was reported as the mean ± S.E.M. for each individual experiment in relation to the control values (vehicle). The level of significance was set to $P < 0.05$. The data were analysed using Student's *t*-test, and 1 or 2-way analysis of variance (ANOVA) followed by Bonferroni's post-hoc test.

Apêndice A – Artigo publicado na revista Neuropharmacology: A novel, potent, oral active and safe antinociceptive pyrazole targeting kappa opioid receptors

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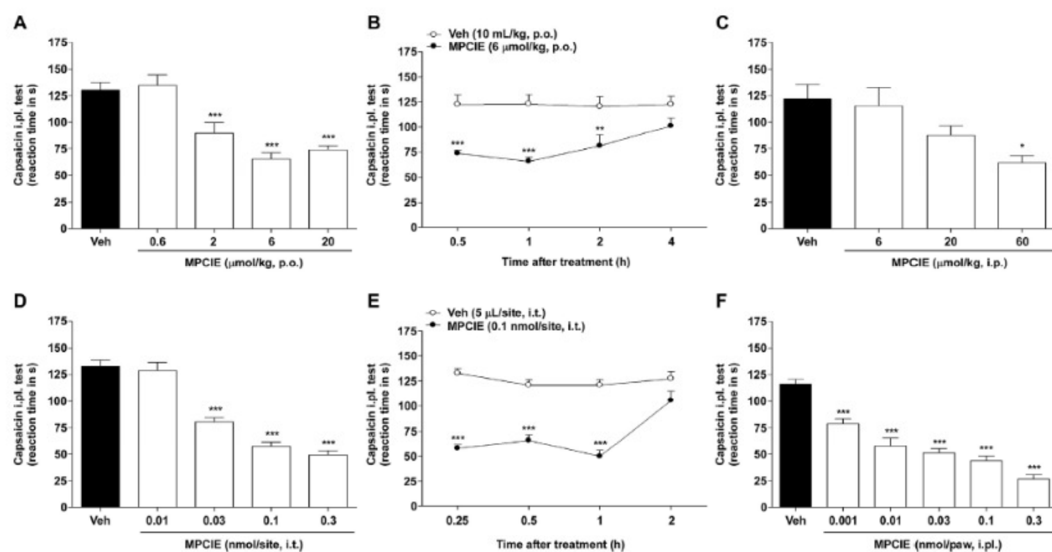

Fig. 2. Antinociception of MPCIE by various administration routes in the mouse intraplantar (i.pl.) capsaicin test (1 nmol/paw, 20 μL). (A) Dose–response (1 h after administration) and (B) time course (6 μmol/kg) curves of MPCIE-induced antinociception after an oral administration (p.o.). (C) Dose–response curve of MPCIE administered intraperitoneally (i.p.) 1 h prior to the capsaicin test. (D) Dose–response (15 min after administration) and (E) time course (0.1 nmol/site, i.t.) curves of MPCIE-induced antinociception after an intrathecal administration (i.t.). (F) Dose–response curve of MPCIE administered intraplantarly 15 min prior to the capsaicin test. Data represent the mean ± S.E.M. of nociception time observed for 5 min after the intraplantar administration of capsaicin ($n = 6–8$). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared to vehicle (veh) treated group; one-way ANOVA (A, C, D, and F) or two-way ANOVA (B and D) followed by Bonferroni's post-hoc test.

G. Trevisan et al. / Neuropharmacology 73 (2013) 261–273

Apêndice A – Artigo publicado na revista Neuropharmacology: A novel, potent, oral active and safe antinociceptive pyrazole targeting kappa opioid receptors

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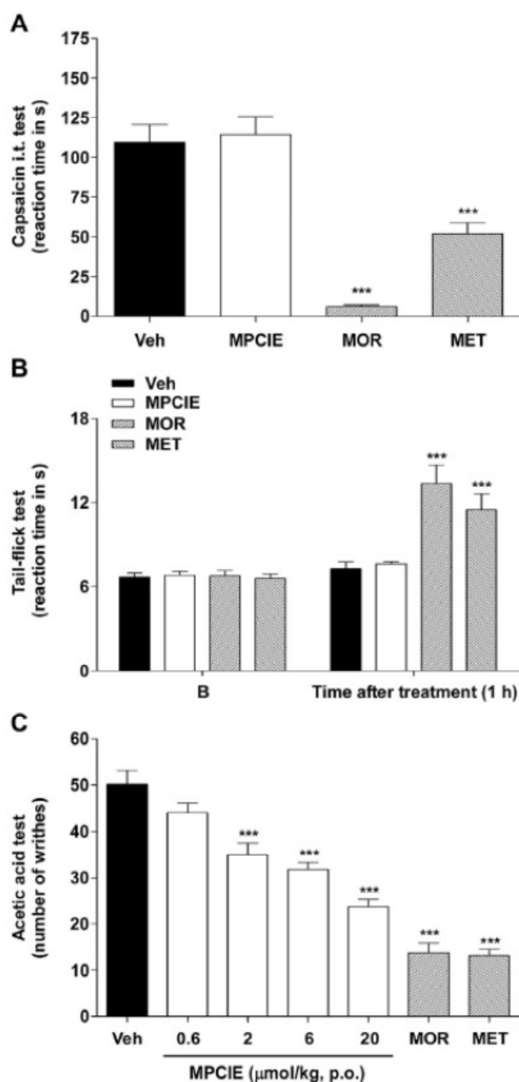

Fig. 3. Effects of an oral administration of MPCIE (6 μmol/kg), morphine (13 μmol/kg), and metamizol (MET, 1 mmol/kg) in (A) intrathecal capsaicin (0.2 nmol/site, i.t.) injection mediated nociception, (B) tail-flick test, and (C) acetic acid-induced writhing evaluated 1 h after drug administration. (C) Inhibition of acetic acid-induced writhing in mice by MPCIE (0.6–20 μmol/kg, p.o.), morphine (MOR, 13 μmol/kg, p.o.), and metamizol (MET, 1 mmol/kg) administered 1 h before the test. Data represent the mean ± S.E.M. of nociception time observed for 5 min after the intrathecal administration of capsaicin (A), the mean ± S.E.M. of reaction time in the tail-flick test (B), and the mean ± S.E.M. of the total number of writhes (C) ($n = 7–8$). Baseline values determinate before the tail-flick test were indicated as "B" in the graph (B). ***$P < 0.001$ compared to vehicle (veh) treated group; one-way ANOVA followed by Bonferroni's post-hoc test (A and C) or two-way ANOVA followed by Bonferroni's post-hoc test (B).

paw) reduced the antinociceptive effects of this compound (Fig. 4H) in the intraplantar capsaicin test. Antagonists alone had no effect on the nociceptive response elicited by the intraplantar injection of capsaicin (Fig. 4).

3.4. MPCIE was able to decrease [^{3}H]-CI-977 binding

Receptor binding assays were performed to assess whether MPCIE directly binds the κ-opioid, α-adrenergic, and the TRPV1 receptors. We have observed a competition between the selective κ-opioid receptor ligand [^{3}H]-CI-977 and MPCIE in a concentration-dependent manner with an IC_{50} value of 0.68 (0.32–1.4) μM (Fig. 5A). However, the specific binding to α_2-adrenergic receptors using [^{3}H]-IDZ or the TRPV1 receptor using [^{3}H]-RTX was not altered (Fig. 5B and C).

3.5. MPCIE possesses antinociceptive effect in clinically-relevant pain models

Once we observed the MPCIE antinociceptive effect on the capsaicin and writhing test; we next investigated its preemptive and curative effect over clinically-relevant pain models. The plantar incision produced a marked mechanical hyperalgesia in the incised paw, observed as an increase in the frequency of response. Curative treatment with MPCIE (6 μmol/kg, p.o., 1 h after incision) reduced mechanical hyperalgesia from 0.5 up to 2 h after treatment (Fig. 6A). In this case, a maximal inhibition was of 67 ± 9% observed at 1 h after treatment. In addition, the preemptive treatment with MPCIE (6 μmol/kg, p.o., 0.5 h before incision) significantly decreased mechanical hyperalgesia from 1 up to 4 h after treatment with a maximal inhibition of 68 ± 5% observed at 2 h after treatment (Fig. 6B).

Moreover, it was possible to observe that intraplantar injection of CFA produced marked mechanical hyperalgesia 48 h after injection. The single oral administration of MPCIE (6 μmol/kg, p.o.) reverted mechanical hyperalgesia induced by CFA from 1 up to 4 h after its administration (Fig. 6C). The maximal effect was observed 2 h after administration with an inhibition of 72 ± 9%.

The partial ligation of mice sciatic nerve produced hyperalgesia, measured 7 days after nerve injury, compared to the sham-operated group (Fig. 6D). MPCIE (6 μmol/kg, p.o.) was markedly effective in reducing the hyperalgesia from 0.5 up to 4 h after treatment, with a maximal inhibition of 84 ± 5%, 1 h after treatment. Moreover, MPCIE did not alter the detection of normal mechanical stimuli assessed in PBS or sham-operated mice (Fig. 6).

3.6. MPCIE did not induce detectable side effects and it is easily absorbed after oral administration

The MPCIE (6 μmol/kg, p.o.) was detected in plasma (0.57 ± 0.04 μmol/mL, $n = 5$), total brain (6.0 ± 0.7 μmol/mg of protein, $n = 5$), and spinal cord (1.4 ± 0.2 μmol/mg of protein, $n = 5$) 1 h after it oral administration.

Since MPCIE may act on the opioid system to produce antinociception, we investigated whether this compound produced common side effects of opioid-like drugs in rodents, such as sedation and constipation. Since motor deficits could interfere in the measurement of nociception, we evaluated the effects of MPCIE on motor performance. The active dose of MPCIE (6 μmol/kg, p.o.) did not alter forced or spontaneous locomotion, as assessed by the rotarod and open-field tests, respectively (Table 1).

We determined whether MPCIE induced sedation, which is indicative of CNS penetration, when administered orally. As shown in Fig. 7A, MPCIE induced sedation only in the highest dose, with ED_{50} value of 927 (393–2188) μmol/kg and an E_{max} of 38 ± 11% (mean ± S.E.M. for % sedation of the highest dose used), respectively. On the basis of the ratio of the sedation or antinociception ED_{50} values, the peripheral restriction index (ED_{50} sedation/ED_{50} nociception) for MPCIE was 126.

Apêndice A – Artigo publicado na revista Neuropharmacology: A novel, potent, oral active and safe antinociceptive pyrazole targeting kappa opioid receptors

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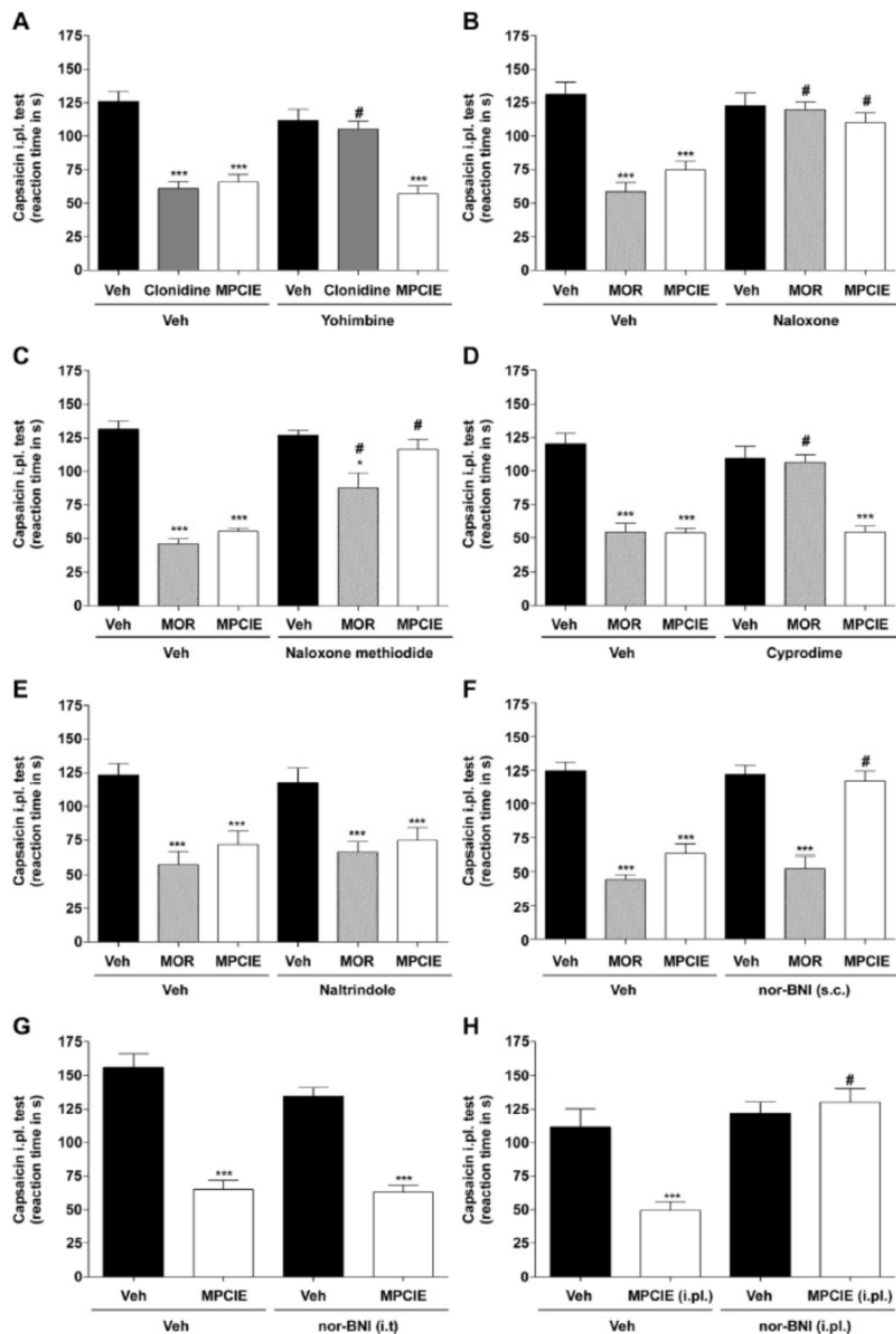

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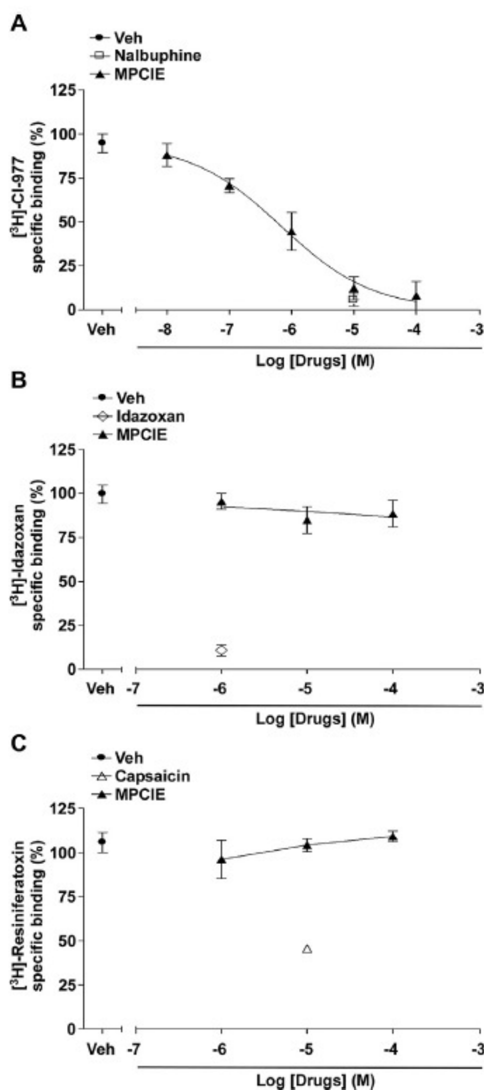

Fig. 5. MPCIE was able to decrease $[^3H]$-CI-977 binding. The effects of MPCIE (0.01–100 μM) on radioligand binding to the (A) kappa-opioid receptor (using $[^3H]$-CI-977 as the radioligand), the (B) α2-adrenergic receptor (using $[^3H]$-Idazoxan as the radioligand), and the (C) TRPV1 receptor (using $[^3H]$-Resiniferatoxin as the radioligand) in vitro. Nalbuphine (10 μM), Idazoxan (1 μM), and capsaicin (10 μM) were used as a positive control in A, B and C, respectively. Each data point represents the mean ± S.E.M. of at least three independent experiments conducted in triplicate, vehicle (veh).

In addition, single oral administration of morphine (13 μmol/kg, p.o.), but not with MPCIE (6, 60, or 600 μmol/kg, p.o.), reduced gastrointestinal transit in comparison with vehicle-treated animals (Fig. 7B). The repeated administration of MPCIE for 7 days did not reduce the gastrointestinal transit of animals (Fig. 7B).

4. Discussion

Pain is a significant health problem, and there is a need for new drugs that provide safe and effective pain management (Bassols et al., 2002; Langford, 2006; Scholz and Woolf, 2002). Pharmacological characterisation of MPCIE showed that this compound possesses antinociceptive activity, which is due to the effects on κ-opioid receptors, with few side effects.

When compared with the novel pyrazole compounds DMPE and MPFE, MPCIE showed marked inhibition of capsaicin-induced nociception. These compounds differed structurally because of the presence of methyl, trifluoromethyl, and trichloromethyl groups attached at the 5 position of DMPE, MPFE and MPCIE, respectively. These pyrazoles were designed by molecular simplification of the lead-like compounds MPCA and MPF4. DMPE, MPFE and MPCIE do not have a hydroxyl group at the 5 position on the pyrazole ring, as opposed to the analogues MPCA and MPF4. The absence of hydroxyl groups and the maintenance of analgesic activity of the 4,5-dihydro-1*H*-pyrazole derivatives indicate that this group has minimal effects on the pharmacophore of this compound class. Moreover, a loss of the hydroxyl group eliminated a chiral centre in the 5 carbon of the pyrazole ring, thus preventing enantiomer formation. In addition, the absence of the hydroxyl group may result in more lipophilic compounds, which may increase the antinociceptive efficacy of DMPE, MPFE and MPCIE compared with MPCA and MPF4.

According to the *rule-of-five* (Lipinski et al., 2001), one can conclude that DMPE, MPFE and MPCIE have properties consistent with good oral absorption since the molecular mass was less than 500 D, the number of hydrogen bond donors was less than 5, the number of hydrogen bond acceptors was less than 10 and the C*LogP* was less than 5. The molecular mass was 154.16 g/mol, 208.13 g/mol and 257.50 g/mol for DMPE, MPFE and MPCIE, respectively, while the number of hydrogen bond donors and hydrogen bond acceptors was 0 and 4, respectively. In addition, the trihalomethylated compounds (MPFE and MPCIE) had better antinociceptive efficacy than DMPE, which has a methyl group attached to the pyrazole ring. Substitution of a methyl group with a trifluoromethyl or trichloromethyl group produced compounds with improved molecular volume and polarisability, which may alter molecular interactions with its putative target. In fact, DMPE had a molecular volume of 143.5 $Å^3$, while MPFE and MPCIE had a molecular volume of 151.4 and 187.0 $Å^3$, respectively. Moreover, in many systems, the substitution of methyl group with a trihalomethyl group results in added lipophilicity, which may lead to easier absorption and transportation of the molecules within biological systems; these effects improve the overall pharmacokinetic properties of the compound (Karthikeyan et al., 2007; Park et al., 2001). The C*LogP* was 0.82 for DMPE, 1.46 for MPFE, and 2.01

Fig. 4. The antinociception produced by orally administered MPCIE was reduced by κ-opioid antagonism. (A) The effects of yohimbine (0.38 μmol/kg, i.p.; $α_2$-adrenoreceptor antagonist) or vehicle (veh) pre-treatment on MPCIE (6 μmol/kg, p.o.) and clonidine (0.38 μmol/kg, i.p.; $α_2$-adrenoreceptor agonist) elicited antinociception in capsaicin test. The effects of (B) naloxone (5 μmol/kg, i.p.; a non selective opioid receptor antagonist), (C) naloxone methiodide (2.1 μmol/kg, i.p.; a non selective opioid receptor antagonist not permeable to the blood–brain barrier), (D) cyprodime (2.3 μmol/kg, i.p.; a selective μ-opioid antagonist), (E) naltrindole (11.1 μmol/kg, i.p.; a selective δ-opioid antagonist), and (F) nor-BNI (13.6 μmol/kg, s.c.; a preferential κ-opioid receptor antagonist) on MPCIE (6 μmol/kg, p.o.) and morphine (MOR, 13 μmol/kg, p.o.) induced antinociception in the intraplantar (i.pl.) capsaicin (1 nmol/paw, 20 μL) test. (G) The effects of intrathecally nor-BNI injection (H, 13.6 nmol/site) or vehicle on MPCIE (6 μmol/kg, p.o.) induced nociception in the mouse i.pl. capsaicin test. (H) The effects of an intraplantar co-administration of nor-BNI (13.6 nmol/paw, i.pl.) or vehicle on MPCIE (0.3 nmol/paw, i.pl.) antinociceptive effect administered 15 min before the i.pl. capsaicin test. Data represent the mean ± S.E.M. of nociception time observed for 5 min after the i.pl. administration of capsaicin to mice ($n = 7–10$). $^{***}P < 0.001$; as compared with vehicle plus vehicle group, $^{\#}P < 0.01$, compared with vehicle plus MPCIE or positive control group; one-way ANOVA followed by Bonferroni's post-hoc test.

Apêndice A – Artigo publicado na revista Neuropharmacology: A novel, potent, oral active and safe antinociceptive pyrazole targeting kappa opioid receptors
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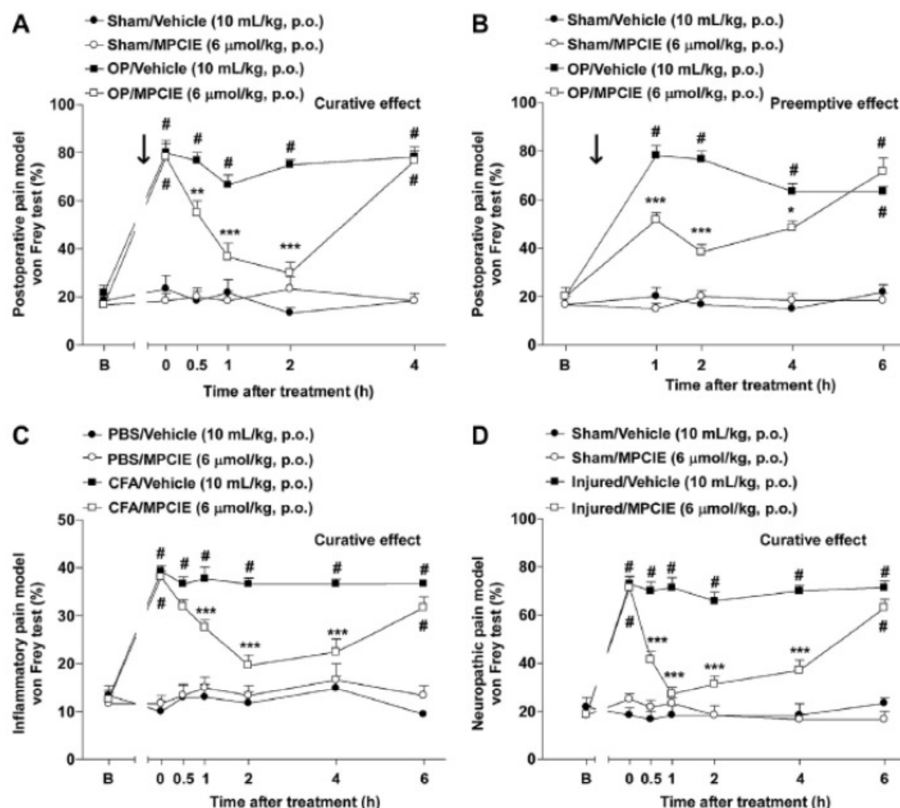

Fig. 6. MPCIE oral treatment reduced the hyperalgesia observed in clinically-relevant pain modes. (A) Curative (treatment 1 h after incision) or (B) preemptive (treatment 0.5 h before incision) effect of MPCIE (6 μmol/kg, p.o.) in a model of postoperative pain (surgical paw incision procedure was indicated with arrows). Operated (OP) or sham (control) animals. (C) Curative MPCIE (6 μmol/kg, p.o.) treatment 48 h after CFA-induced inflammatory nociception, PBS injected animals were used as control. (D) Curative effect of MPCIE (6 μmol/kg, p.o.) treatment 7 days after neuropathic pain model induced by partial sciatic nerve ligation (injured animals), sham animals were used as control. Basal measures (B) were observed before the operative procedure, or CFA and PBS injection. Data represent the mean ± S.E.M. of frequency of response to a von Frey filament (0.09 g) ($n = 7–10$). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared to vehicle (veh) treated group sham operated or PBS i.pl. injected; two-way ANOVA followed by Bonferroni's post-hoc test.

for the trichloromethylated compound MPCIE. The lipophilicity of MPCIE may be related to its superior antinociceptive activity when compared with its analogues DMPE and MPFE.

A comparison of the various administration routes revealed that MPCIE might have good biodisponibility when administered orally. In fact, it is easily absorbed after oral administration. The formation of a potent and safe metabolite after the p.o. injection of MPCIE by first-pass hepatic metabolism of this compound may explain the better potency observed after p.o. administration when compared to the i.p. route. In addition to its efficacy to induce antinociception when administered directly into the periphery (i.pl.) or into the central nervous system (i.t.), MPCIE seems to be more potent and efficacious to induce antinociception when administered peripherally. Thus, we performed additional nociception tests to elucidate the mechanism of systemically administrated MPCIE-induced antinociception. First, we performed a tail-flick test, regarding the sensitivity to clinically used analgesic/anti-inflammatory drugs this test is sensitive to opioids and atypical NSAIDs (e.g. NSAIDs that possess poor anti-inflammatory effect, such as metamizol) and usually insensitive to typical NSAIDs (e.g. NSAIDs that possess good anti-inflammatory effect, such as indomethacin) and steroids (Negus et al., 2006). Oral administration of MPCIE did not produce antinociception in the tail-flick test. This result suggests that this compound does not act on the central nervous system since an analgesic drug must cross the blood–brain barrier and penetrate the CNS to be effective in this test. In fact, the tail-flick test may be used as an index of blood–brain barrier penetration

Table 1
The effects of MPCIE (6 μmol/kg, p.o.) and vehicle on forced (rotarod test) and spontaneous (open-field test) locomotor activity in mice 1 h after treatment.

Treatment	Rotarod		Open-field	
	First fall (s)	Falls	Crossing	Rearing
Vehicle	191 ± 24	2.3 ± 0.7	47 ± 7	9 ± 1
MPCIE	149 ± 36	1.2 ± 0.6	52 ± 4	12 ± 1

Significant differences were not observed between groups (Student's *t*-test). Data represent the mean ± S.E.M of the latency until the first fall and the total number of falls in the rotarod test and the number of crossing and rearing in the open-field test ($n = 7–9$).

Apêndice A – Artigo publicado na revista Neuropharmacology: A novel, potent, oral active and safe antinociceptive pyrazole targeting kappa opioid receptors
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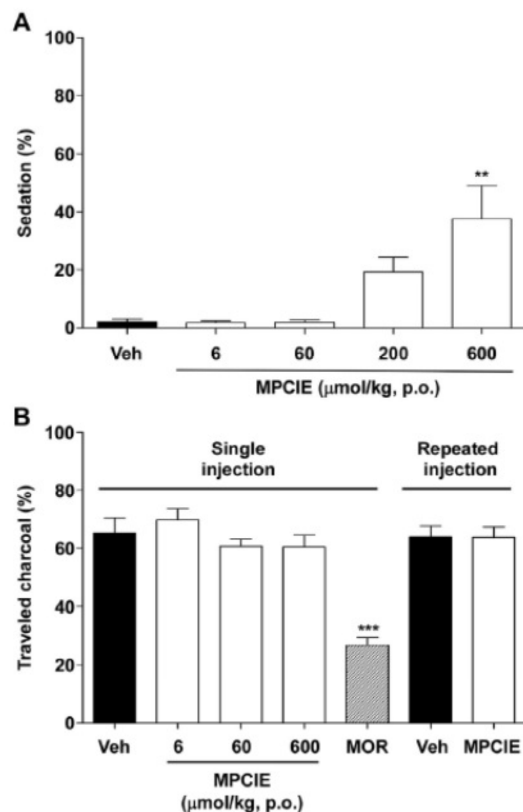

Fig. 7. MPCIE oral administration did not induce sedation or alter the gastrointestinal motility in mice. (A) MPCIE induced sedation only at high dose in the mice platform sedation test. (B) The effects of MPCIE (6, 60, or 600 μmol/kg, p.o.), morphine (13 μmol/kg, p.o.), and vehicle after single administration, or after repeated administration for 7 days of MPCIE (6 μmol/kg, p.o.) and vehicle on gastrointestinal motility in mice. Data represent the mean ± S.E.M. of the % of sedation (A) or of the distance charcoal travelled (%) through the small intestine (B) (n = 5–7). ***P < 0.001, when compared with vehicle (veh) treated animals; one-way ANOVA followed by Bonferroni's post-hoc test.

for peripherally administered opioid agonists (Vanderah et al., 2008). Corroborating this data, our results demonstrated that orally administered MPCIE reduced capsaicin-induced nociception when this algogen was peripherally (i.pl.), but not centrally (i.t.), injected. Furthermore, in the capsaicin test, MPCIE produced remarkable antinociception when locally administered into the ipsilateral paw, but not when administered into the contralateral paw, indicating that these effects were local and not systemic. Finally, we investigated the effects of MPCIE in the acetic acid test, which, as opposed to the tail flick test, is used to detect antinociceptive activity of drugs acting on the peripheral, spinal and supraspinal level (Hardy et al., 1989; Porreca et al., 1987). In agreement with the hypothesis that MPCIE acts mainly peripherally, oral administration of MPCIE induced antinociception in this test. As our preliminary pharmacokinetic investigation demonstrated, MPCIE seems to cross the blood brain barrier. Thus, possibly this compound may have different affinities at central and peripheral kappa receptors, what may explain some of its preferential peripheral action.

Using in vitro and in vitro methods, we further investigated the mechanism responsible for MPCIE-induced antinociception. As MPCIE reduced the nociception induced by the TRPV1 receptor agonist capsaicin, an obvious question to be addressed was whether MPCIE binds the TRPV1 receptor. We found that MPCIE did not alter the specific binding of [^{3}H]-resiniferatoxin, demonstrating that this compound did not interact directly with the TRPV1 receptor. Previous studies have demonstrated that α_2-adrenoceptors and opioid receptors are involved in the antinociceptive effects of the lead-like pyrazole MPCA and MPF4 (Godoy et al., 2004; Milano et al., 2008a, 2008b). Using antagonists in vivo and radioligand assays, we clearly showed that the α_2-adrenoceptor did not mediate the antinociceptive effects of MPCIE.

On the other hand, the antinociceptive effects of MPCIE were mostly mediated by peripheral opioid receptors, as these were fully blocked by naloxone and its analogue, which does not cross blood–brain barrier. In contrast to MPCIE, naloxone methiodide failed to fully antagonise the antinociceptive effects of morphine, which mainly exerts central effects (Labuz et al., 2007). In addition, the selective δ and μ-opioid antagonists, failed to reverse the antinociceptive effect of MPCIE. However, the preferential κ-opioid receptor antagonist nor-BNI given systemically or peripherally reversed the antinociceptive effects of MPCIE. Furthermore, we demonstrated that MPCIE directly interacted with κ-opioid receptors, since it displaced binding of the selective ligand [^{3}H]-CI-977. However, nor-BNI failed to antagonise the antinociceptive effects of MPCIE when the antagonist was administered intrathecally, demonstrating that the antinociceptive effects of MPCIE could be mainly mediated by stimulation of the peripheral κ-opioid receptors. Accordingly, some studies have shown that opioid-induced antinociception may also be mediated by the activation of opioid receptors located outside the central nervous system (DeHaven-Hudkins and Dolle, 2004; Zhang et al., 1998).

Interestingly, besides the antinociceptive effect of MPCIE in capsaicin and whiriting testes oral administration of this compound was also able to reduce the hyperalgesia observed in a model of postoperative, inflammatory, and neuropathic pain in mice. Interestingly, in the model of postoperative pain we observed that this compound exerted either pre- or postoperative effects. Similar results were observed for the metamizol (dipyrone) and morphine in a previous work (Milano et al., 2008b). These results were the significant importance because all these categories of pain frequently have a complicated treatment and the available drugs are not effective or produce considerable adverse effects (Labianca et al., 2012).

MPCIE does not cause constipation at effective doses after single or repeated administration. This effect was previously observed for opioid kappa agonists (also peripheral kappa agonists), but it is more frequently induced by morphine administration (Ballantyne, 2007; Gallantine and Meert, 2008; Mangel and Hicks, 2012). There is potential for the therapeutic application of opioid κ-agonists as analgesics (Hunter et al., 1990; Vonvoigtlander and Lewis, 1988); however, these compounds present some centrally acting side effects, such as sedation (Pande et al., 1996) and also dysphoria (Land et al., 2008). In this context, a common strategy to reduce the centrally acting side effects of drugs that have beneficial peripheral actions is to restrict CNS penetration. Thus, a class of peripherally selective κ-agonists, such as asimadoline (Barber and Gottschlich, 1992) and D-amino acid tetrapeptide (Vanderah et al., 2008), hold great promise as novel analgesics that lack undesirable side effects (Barber and Gottschlich, 1992; Pande et al., 1996; Vanderah, 2010). MPCIE is similar to this class of compounds has a higher selective restriction index (126), which is higher than that of other peripheral κ-opioid agonists (Kumar et al., 2005). Furthermore, at doses that produce maximal antinociceptive effects, MPCIE does not

Apêndice A – Artigo publicado na revista Neuropharmacology: A novel, potent, oral active and safe antinociceptive pyrazole targeting kappa opioid receptors

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cause motor impairment, indicating that the antinociceptive effects and the motor impairment/sedative activity are dissociated from centrally acting κ-opioid agonists (Vanderah et al., 2008, 2004). Thus, this compound has ideal pharmacologic characteristics, a lack of detectable side effects and efficacy in clinically-relevant pain models when administered systemically.

5. Conclusions

In summary, MPClE shows potent antinociceptive activity, demonstrating that molecular simplification is an efficient method to obtain novel and effective pyrazole derivatives. The analgesic effects elicited by MPClE seem to be mediated via kappa opioid receptors. Thus, these data suggest that MPCIE may produce antinociceptive effects without considerable side effects and that it may represent a better analgesic therapy in comparison with centrally acting opioids. This MPCIE may be used as a mild analgesic prototype in the development of novel clinically active analgesics.

Conflict-of-interest disclosure

The authors declare no competing financial interests.

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Apêndice A – Artigo publicado na revista Neuropharmacology: A novel, potent, oral active and safe antinociceptive pyrazole targeting kappa opioid receptors

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