SERVIÇO PÚBLICO FEDERAL

UNIVERSIDADE FEDERAL DE UBERLÂNDIA

INSTITUTO DE BIOTECNOLOGIA

PROGRAMA DE PÓS-GRADUAÇÃO EM GENÉTICA E BIOQUÍMICA

AVALIAÇÃO DO POTENCIAL TERAPÊUTICO DO ANTAGONISMO DOS RECEPTORES AT2 DE ANGIOTENSINA II NA GOTA

Aluno: Thiago Neves Vieira

Orientadora: Profa. Dra. Cássia Regina da Silva

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COMISSÃO EXAMINADORA

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Profa. Dra. Cássia Regina da Silva

DEDICATÓRIA

Se tiver o hábito de fazer as coisas com alegria, Raramente encontrará situações difíceis.

Robert Baden-Powell

Dedico este trabalho aos meus pais, Rivail Francisco Vieira e Joana Darc Freitas Neves Vieira, pelo apoio incondicional em todos sentidos possíveis. A todos os professores que instigaram meu conhecimento e me guiaram em minha jornada acadêmica e que lutam diariamente para manter as pesquisas em seus laboratórios.

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"*Se você não se sente a altura, suba até ela*" – Masashi Kishimoto

SUMÁRIO

CAPÍTULO I

FUNDAMENTAÇÃO TEÓRICA

A artrite gotosa é uma doença metabólica persistente que atinge articulações como a metatarso-falangeana e tíbio-tarsal. Sua prevalência varia de país para país, sendo mais comum em homens em comparação a mulheres, sua incidência e ocorrência aumenta em pessoas acima de 40 anos (JOSHI, 2012). Além disso diversos estudos mostram um aumento da prevalência da doença ao longo dos anos, devido a mudanças de hábitos alimentares e aumento de síndromes metabólicas (CHEN-XU et al., 2019; DEHLIN et al., 2016; RAI et al., 2016).

A gota é caracterizada pela deposição de cristais de urato monossódico (MSU) em articulações pequenas como a tíbio-tarsal, essa deposição leva a uma resposta inflamatória muito dolorosa. A primeira fase da gota ocorre em indivíduos com hiperuricemia. Pode ser dividida em quatro fases: hiperuricemia assintomática, crises agudas, período intercrítico e gota tofácea crônica (CAMPION, 1987). O ataque agudo é reconhecido como uma das experiências mais dolorosas conhecidas, no mesmo nível das dores do parto e de cólicas viscerais (REES et al., 2014). Os tratamentos muitas vezes são contraindicados pela presença de comorbidades nos indivíduos acometidos, e a prevenção se resume a um controle de dieta, assim, a gota ainda é uma doença de difícil tratamento, visto que os mecanismos de ação não estão completamente elucidados.

Ao que se refere as comorbidades, a maioria dos indivíduos com gota sofrem ainda de hipertensão, chegando a aproximadamente 74% dos indivíduos (CHOI et al., 2012; ZHU et al., 2012). O tratamento de hipertensão, em sua maioria, consiste no uso de fármacos inibidores da enzima conversora de angiotensina (iECA) e dos bloqueadores dos receptores AT1 para angiotensina II (BRAT1) que podem agir via modulação do sistema renina-angiotensina (CHOI et al., 2012). Contudo, o uso de tais fármacos aumenta o risco de se desenvolver um ataque agudo de gota, esse efeito está em parte, relacionado a regulação do sistema das cininas (SILVA et al., 2014). Ainda assim, pouco se sabe sobre o

possível papel do sistema das angiotensinas (XU et al., 2013).

A associação da gota e a hipertensão já foi demonstrada através de modelos animais de roedores, onde nosso grupo sugeriu que a administração de uma baixa concentração de cristais de MSU, incapaz de causar dor e inflamação por si só, quando combinada com fármacos anti-hipertensivos como os anteriormente citados, leva a um quadro de sintomas inflamatórios semelhante ao do ataque agudo de gota (Silva et al., 2016).

Os RAT2 são receptores acoplados a proteína G, eles são expressos em diversas células do corpo humano como células endoteliais (Pueyo & Michel, 1997), macrófagos (Shepherd et al., 2018 A, B), fibroblastos (Anand et al., 2013), sinoviócitos, neurônios sensoriais (Terenzi et al., 2017) e condrócitos (Tsukamoto et al., 2013), células estas que também estão presentes no ambiente articular (Dalbeth et al., 2019). Estudos recentes apontam o antagonismo do receptor AT2 como um alvo promissor no tratamento de doenças dolorosas crônicas e neuropáticas (Rice et al., 2014; Smith et al. 2016; Shepherd et al., 2018 A, B).

O antagonista dos RAT2, PD123319, ou ainda EMA401 é uma imidazopiridina (4,5,6,7 tetrahidro,1H-imidazo[4,5-C]-piridina) contendo 4 (dimetilamino)-3-metilbenzil, difenilacetil e carboxi, nas posições 1, 5 e 6 respectivamente, sua forma molecular é $C_{31}H_{32}N_4O_3$ (National Center for Biotechnology Information, 2022). O uso do antagonista do RAT2 como tratamento já foi demonstrado em um estudo clínico de fase II, onde foi utilizado EMA401 por 4 semanas em pacientes portadores de neuralgia pós-herpético realizado por Rice e colaboradores (2014), o tratamento levou a uma redução na dor neuropática dos pacientes. Corroborando esses dados, estudos pré-clínicos realizados por Shepherd et al (2018A;B) demonstraram que o bloqueio do RAT2 com o antagonista PD123319 promoveu analgesia em modelo experimental de dor neuropática induzida por transecção de nervo ciático. Adicionalmente, este estudo também verificou que a Angiotensina-II parece ter papel fundamental na manutenção de quadros de dor neuropática por modular positivamente os canais iônicos TRPA1 presentes nos neurônios nociceptivos aferentes primários via ação RAT2 em macrófagos. Portanto o RAT2 se demonstra como um alvo

terapêutico interessante a ser explorado no processo inflamatório da gota.

1.1 Dor

O conceito de dor, recentemente atualizado pela Associação Internacional para o Estudo da Dor (IASP), abrange uma experiência sensorial e emocional desagradável associada a potencial ou real dano tecidual (RAJA et al., 2020). A dor é também um importante mecanismo de sobrevivência, que pode ser iniciada em qualquer parte do corpo através de um amplo sistema sensorial. O papel protetor da dor é mais evidente em indivíduos com insensibilidade congênita à dor, uma condição genética rara que resulta na incapacidade de detectar danos nos tecidos ou estímulos nociceptivos (COX et al., 2006). Essa resposta normalmente protetora, que está ausente nesses indivíduos, leva a lesões frequentes e resulta, muitas vezes, em taxas de mortalidade mais altas no início da vida (BENNETT e WOODS, 2014).

A dor pode ser classificada em dois tipos, aguda e crônica, sendo que a dor aguda tem um importante papel na proteção tecidual e pode desaparecer antes mesmo do restabelecimento do tecido lesado. Já a dor crônica é prejudicial ao corpo, acarretando sofrimento e incapacidade, com duração mínima de três meses, porém, podendo acompanhar o indivíduo pelo resto da sua vida (WOOLF, 2010; MILLAN, 2002).

Os estudos sobre a dor em humanos são difíceis de realizar, são subjetivos e limitados por considerações éticas, levando ao uso generalizado de animais como modelos para estudar a dor, sendo as espécies mais comumente utilizadas ratos e camundongos (MOGIL, 2009). No entanto, com o uso de modelos animais, surgem desafios relacionados com a quantificação de respostas comportamentais que poderiam ser consideradas equivalentes a dor em humanos. É usado então o termo nocicepção, que tem como definição somente o estímulo doloroso, não levando em consideração fatores emocionais, assim, inclui as vias neuroanatômicas, mecanismos neurológicos e receptores que são específicos para detectar estímulos nocivos. Logo, em animais é avaliada a nocicepção (KANDEL et al., 2003).

A detecção da nocicepção é feita por neurônios especializados,

chamados nociceptores (SHERRINGTON, 1906). Estes neurônios estão presentes no sistema sensorial periférico, porém seu corpo celular se encontra na raiz do gânglio dorsal (exceto na face) no sistema nervoso central. Devido a variedade de estímulos nocivos, também existem diferentes tipos de nociceptores, que podem ser divididos em três classes: tipo Aδ, fibras mielinizadas com médio diâmetro, tipo Aα e Aβ, mielinizadas com grande diâmetro e tipo C, delgadas e amielínicas. As fibras do tipo Aδ, podem ainda ser subdivididas em: Tipo I, especializadas em respostas mecânicas intensas e estímulos químicos e Tipo II, especializadas em respostas térmicas nocivas (JULIUS e BASBAUM, 2001; MEYER et al., 2006; DUBIN e PATAPOUTIAN, 2010).

A nocicepção se da através de quatro passos: Transdução, onde ocorre a ativação dos neurônios sensoriais por um estímulo nocivo, levando ao influxo de sódio e cálcio via canais TRP, gerando potencial de ação; Transmissão, o potencial de ação corre por neurônios de primeira ordem e liberam neurotransmissores que ativam neurônios de segunda ordem no dorso da medula espinhal, onde neurônios ascendentes levarão a resposta nociva aos neurônios de terceira ordem no encéfalo; Modulação, no encéfalo e até em fibras descedentes há vários moduladores endógenos, como o humor e contexto da situação do indíviduo que podem atenuar ou aumentar a percepção da dor; Percepção, após a percepção da dor pelo indíviduo neurônios descendentes são responsáveis por tomar uma ação a fim de se proteger do estímulo nocivo (BASBAUM et al., 2009).

A dor é considerada um problema de saúde frequente que causa prejuízos econômicos e pessoais à população. Dores agudas de forma repetida podem levar a hiperalgesia mecânica, uma sensibilidade aumentada à dor. A inflamação articular frequentemente leva a hiperalgesia, na artrite, os nervos articulares tornam-se sensibilizados, produzindo dor aguda (VON BANCHET et al., 2007). A inflamação na gota se inicia através do reconhecimento dos cristais de MSU pelo sistema imune, porém essa resposta pode ser modulada, a fim de reduzir a inflamação e dor que acomete os pacientes, indicando assim uma necessidade de elucidar melhor seus mecânismos pró-inflamatórios (SO e MARTINON,

2017).

As desordens inflamatórias como as artrites estão entre as situações crônicas que mais frequentemente afetam a população mundial. Têm um forte impacto na qualidade de vida, no uso de recursos, cuidados médicos e na economia de um país. A gota ou artrite gotosa foi uma das primeiras artrites a ser clinicamente descrita e está entre as artrites de maior prevalência, além disso há um aumento do número de pessoas com gotas ao longo de tempo (RICHETTE e BRADIN, 2010; NEOGI, 2011; JOSHI, 2012; CHEN-XU et al., 2019).

1.2 Artrite Gotosa

O ataque agudo de gota, se inicia geralmente em articulações dos membros inferiores como a tíbio-tarsal, levando a indíviduo a sentir muita dor articular por um período de 7 a 14 dias (TAYLOR et al., 2015; DALBETH et al., 2021). A articulação afetada fica edemaciada, há grande presença de leucócitos no fluido sinovial e a pele circundante torna-se vermelha ou púrpura, rígida e brilhante, com uma sensação de calor e percepção de dor de forte intensidade (CHOI et al., 2005). É caracterizada pela deposição articular de cristais de urato monossódico (MSU), esses cristais são a forma sólida do ácido úrico, produto final do metabolismo das purinas, que pode se acumular e cristalizar em tecidos orgânicos (GEORGE, 2009).

A gota pode ser dividida em quatro fases: primeiro a hiperuricemia assintomática, onde o indivíduo apresenta altos índices de ácido úrico no sangue, porém não há nenhum tipo de sintoma, após muitos anos apresentando esse quadro de hiperuricemia o indivíduo pode vir a desenvolver ataques agudos. A segunda fase ocorre no primeiro ataque agudo de gota na qual os pacientes relatam picos de dor principalmente durante o período da noite e nas articulações do metatarso e metacarpo, porém esse ataque cessa sozinho até o período de 14 dias no máximo. A terceira fase, chamada de período intercrítico, ocorre entre crises de ataque agudo, totalmente assintomático e de duração variada, se não tratada a hiperuricemia o período entre os ataques agudos se torna cada vez menor. A quarta fase, chamada de gota tofácea é o estágio

crônico e mais avançado da doença, nessa fase há o desenvolvimento de aglomerados de cristais de MSU e células do sistema imune chamados de tofos, que dão o nome dessa condição, os indivíduo acometidos tem perda de função e deformações nas articulações afetadas (CAMPION et al., 1987; DALBETH et al., 2021).

A hiperuricemia (nível de ácido úrico acima de 7 mg/L de sangue), condição onde as concentrações de ácido úrico estão acima do comum, aumenta os riscos de se desenvolver gota, a sua alta concentração no sangue e estágio crônico estão correlacionadas ao desenvolvimento de doenças cardiovasculares, doenças renais e hipertensão (FEIG et al., 2008; KUTZING e FIRESTEIN, 2008; TERKELTAUB et al., 2006; DALBETH et al., 2021). O risco de se desenvolver gota aumenta com a permanência da hiperuricemia e com os níveis de ácido úrico, porém, esta condição nem sempre evolui para o desenvolvimento da doença, não se sabe ainda ao certo o porquê.

A segunda fase da doença, o ataque agudo de gota, é o foco do nosso trabalho, nessa fase há grande presença de dor e inflamação, além de uma grande sensibilização do local afetado, ela é descrita pelas pessoas acometidas como lascinante, intensa, ardente e latejante de duração variável (TAYLOR et al., 2015). No modelo de ataque agudo de gota em camundongos a dor mecânica e térmica pode ser acessada em modelos animais através de estímulos mecânicos com uso dos filamentos de vonfrey, além da avaliação da nocicepção espontânea e nocicepção térmica. A inflamação é avaliada através da avaliação da espessura da articulação, bem como através da coleta de líquido sinovial articular local, para avaliar células inflamatórias presentes no ataque agudo de gota, como neutrófilos e IL-1β.

A gota é considerada uma doença tratável, os tratamentos consistem em: reduzir os níveis de ácido úrico na terceira fase da doença, utilizando fármacos como alopurinol, febuxostat, probenecida, lesinurad e pegloticase; e reduzir a dor e inflamação do ataque agudo de gota, com fármacos como anti-inflamatórios não esteroidais (AINEs), colchicina, corticosteróides e inibidores da IL-1, (SCHLESINGER, 2017). O desenvolvimento de novas terapias é importante e necessário, pois o controle da dieta e mudança de hábitos ainda que eficientes,

possuem uma baixa adesão pela população, não resolvendo o problema por si só (SO e MARTINON, 2017; YOKOSE et al., 2021). Os fármacos atualmente disponíveis, possuem muitas contraindicações, devido a grande presença de comorbidades e possíveis interações medicamentosas decorrentes da doença. Por exemplo, a análise dos registros médicos de 575 pacientes diagnosticados com gota nos Estados Unidos, apontaram que mais de 88% apresentavam pelo menos uma condição patológica das que são consideradas comorbidades comuns a gota como doenças cardiovasculares, diabetes tipo 2, hiperlipidemia, síndrome metabólica, doenças crônicas nos rins e nefrolitíase (KEENAN et al., 2011). Os efeitos colaterais dos fármacos disponíveis também são outra preocupação, entre eles ulceração peptídica, intolerância gástrica, sobrecarga e alterações na função renal em virtude das doses terapêuticas serem próximas as doses tóxicas (SCHLESINGER, 2017). Outros fármacos como as terapias de inibição da IL-1 não são recomendadas como primeira opção de anti-inflamatórios (SO e MARTINON, 2017). Portanto a gota se mostra como uma doença com opções de tratamento, que ainda carece de novas opções de fármacos, ainda melhor caso atuem alvos diferentes dos atualmente explorados.

Ao que se refere as comorbidades, a maioria dos afetados pela gota sofrem ainda de hipertensão, chegando a aproximadamente 74% dos indivíduos (CHOI et al., 2012; ZHU et al., 2012). O tratamento de hipertensão inclui na grande parte dos casos o uso de fármaco inibidores da enzima conversora de angiotensina (iECA) e os bloqueadores dos receptores AT1 para angiotensina II (BRAT1). Esses fármacos atuam no sistema renina angiotensina bloqueando a ação do receptor para Angiotensina II tipo 1 e a clivagem da Angiotensina I em Angiotensina II. Contudo, o uso de tais fármacos pode levar ao desenvolvimento de ataques agudos de gota, esse efeito está em parte relacionado a regulação do sistema das cininas (XU et al., 2013; SILVA et al., 2015).

1.3 Sistema Renina-Angiotensina

O Sistema Renina Angiotensina tem como precursor o Angiotensinogênio, uma glicoproteína produzida pelo fígado que é clivado por hidrólise pela enzima Renina em Angiotensina I, um decapeptídeo inativo. A Angiotensina I por sua

vez é clivada pela ECA em Angiotensina II. Além da Angiotensina II vários outros produtos são formados nesta via, como Angiotensina (1-7), Angiotensina (1-9), Angiotensina III e Angiotensina IV. Os outros produtos possuem meios distintos de formação, como a Angiotensina (1-7) que é formada pela hidrólise da Angiotensina II pela ECA2, mas também pode ser formada pela hidrólise da Angiotensina I por prolil-endopeptidases, neutro-endopeptidases e timet-oligopeptidases. Além da hidrólise da Angiotensina II a ação de carboxi-peptidases e prolil-endopeptidases podem contribuir para a formação de Angiotensina (1-7). A formação de Angiotensina III e IV é dependente de aminopeptidases e a clivagem é feita pela ECA (BADER., 2010).

Abaixo a Fig. ilustra as vias descritas acima:

Figura 1: Sistema Renina-Angiotensina

Adaptada de: Passos-Silva DG (2015)
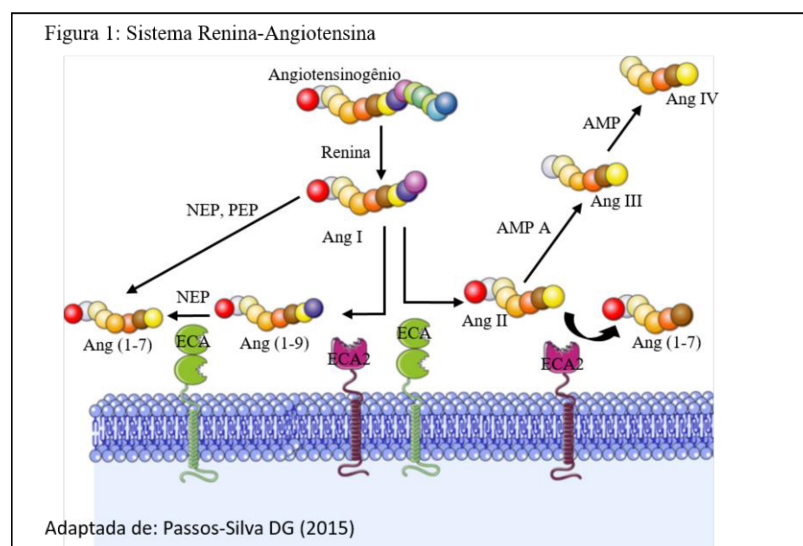
Figura 1. Visão simplificada do sistema de cascata Renina-Angiotensina. Abreviações: Ang I, angiotensina I; Ang II, angiotensina II; Ang III, angiotensina III; Ang IV, angiotensina IV; Ang (1-7), angiotensina (1-7); Ang (1-9), angiotensina (1-9); AMP, Aminopeptidase; ECA, enzima conversora de angiotensina; ECA2, enzima conversora de angiotensina II; NEP, Neutro-endopeptidase; PEP, Propil-endopeptidase.

O sistema Renina-Angiotensina desempenha um papel crucial na homeostase hidroeletrolítica e cardiovascular, atuando através da vasoconstrição (BADER, 2010; KOBORI et al., 2007).

Os receptores AT1 quando estimulados pela Angiotensina II leva a

8

vasoconstrição, anti-natriurese, secreção de aldosterona, ativação do sistema nervoso simpático, crescimento e diferenciação celular (CAREY, 2013). Apesar do RAT2 ter sido descoberto a muito tempo, diferente do receptor AT1, as ações da sua ativação não são totalmente claras. A estimulação dos receptores AT2 leva a um bloqueio da ação vista pela ativação do RAT1 pela angiotensina II, seus efeitos descritos envolvem inibição da proliferação e diferenciação celular, promoção de vasodilatação em oposição ao efeito de vasoconstrição da ativação do RAT1, regulação dos níveis de NO nos rins, portanto desempenhando funções principalmente na regulação homeostática do sistema cardiovascular, rins e cérebro (Matavelli & Siragy, 2015).

Para o tratamento da hipertensão são usados os bloqueadores de receptores AT1 (BRAT1) como a valsartana. Além disso também são usados os inibidores da enzima conversora de angiotensina (iECA) como enalapril, para evitar a clivagem de Angiotensina I em Angiotensina II. Acredita-se que os BRAT1 quando usados levam a um aumento de disponibilidade de angiotensina II, pois ela não irá ativar receptores AT1, tendo assim uma maior disponibilidade para ativar outros alvos, como os receptores AT2 (CAREY, 2005). Já os iECA bloqueiam a ECA1 deixando uma maior disponibilidade de Angiotensina I para ser clivada pela ECA2 em Angiontensina 1-9, que por sua vez também pode ativar receptores AT2 (CHA et al., 2018).

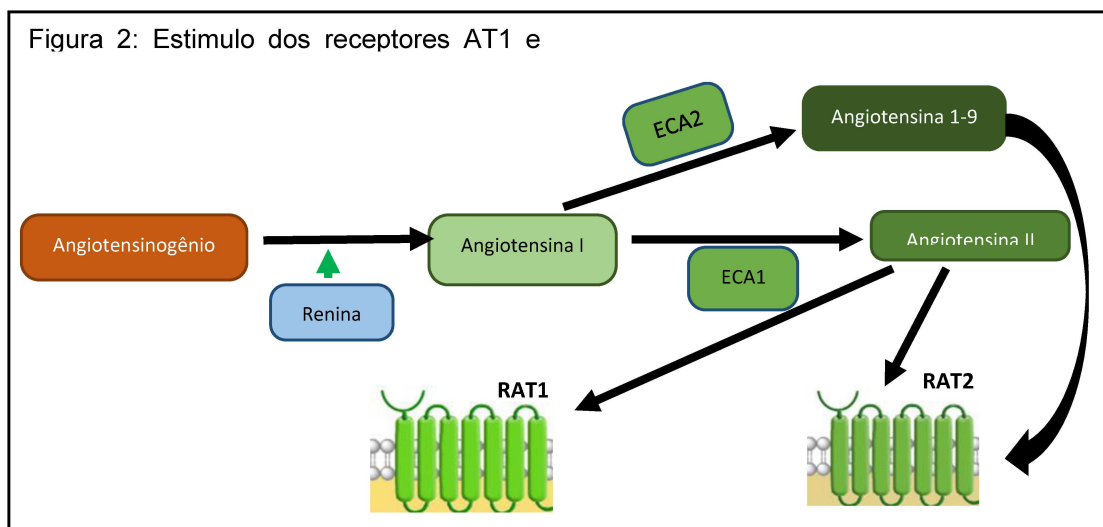Abaixo um esquema que ilustra possíveis ativadores dos receptores AT1 e AT2

Figura 2: Estimulo dos receptores AT1 e

Figura 2. Visão simplificada das vias de estímulo aos receptores AT1 e AT2. Abreviações: RAT1, Receptor para angiotensina tipo 1; RAT2, receptor para angiotensina tipo 2; ECA, enzima conversora de angiotensina; ECA2, enzima conversora de angiotensina tipo 2.

Além do papél dos receptores AT2, crucial na regulação homeostática já conhecido, estudos recentes mostram um papel também na dor.

1.4 Receptores para angiotensina II tipo 2

O sistema renina-angiotensina tem dois principais receptores que são ativados pelas angiotensinas, os receptores para angiotensina tipo 1 e tipo 2. Ambos receptores pertencem a família 1 de receptores acoplados a proteína G (VARGAS et al., 2022). O receptor AT2 é uma proteína de membrana intergral composta por 363 aminoácidos de peso molecular 41 kDa, sua expressão é codificada pelo gene 186 em humanos, localizado no cromossomo X (KAMBAYASHI et al., 1993; National Center for Biotechnology Information, 2022). Sua expressão se encontra em diversas células também presentes no ambiente articular, como neurônios sensoriais periféricos, células endoteliais, sinoviócitos e macrófagos periféricos (Pueyo & Michel 1997; Terenzi et al. 2017; Shepherd et al. 2018 A).

Apesar das semelhanças do RAT2 com o RAT1, suas diferenças são notáveis, enquanto a ação do RAT1 é conhecida pelo papél na regulação homeostática principalmente de rins e sistema cardiovascular a muito tempo, o RAT2 se mostra com funções ainda muito controversas e apenas recentemente descobertas (RANJIT et al., 2021). A ativação dos RAT2 é responsável por inibir proliferação e diferenciação celular, promoção de vasodilatação, apoptose, entre outras, quanto a morte celular programada, ela pode desempenhar um papel importante na biologia e fisiopatologia do desenvolvimento. Mutações no seu gene estão associadas à deficiência cognitiva ligada ao cromossomo X. A síndrome respiratória aguda grave por coronavírus (SARS-CoV) e SARS-CoV-2 resulta na regulação negativa dos receptores da ECA2, desencadeando lesões inflamatórias graves principalmente em pulmões. A reação inflamatória parece ser mediada por derivados da angiotensina II, incluindo o receptor AT2 da

angiotensina, que foi regulado positivamente em amostras de lavado broncoalveolar de pacientes com doença de coronavírus 2019 (COVID19) (National Center for Biotechnolgy Information, 2022). A expressão dos RAT2 também é regulada positivamente pela IL-1β, a principal citocina pró-inflamatória do ataque agudo de gota (KAMBAYASHI et al., 1993).

Na literatura é demonstrado efeito do antagonismo do RAT2 em diversos modelos nociceptivos e inflamatórios, entre eles modelos de dor neuropática (SMITH et al., 2013; SHEPHERD et al., 2018 B), modelo de dor óssea induzida por câncer de próstata (MURALIDHARAN et al., 2014), modelo de hipersensibilidade mecânica por vestibulodinia provocada (CHAKRABARTY et al., 2018). Além disso um trial clínico de fase 2 demonstrou que o bloqueio do RAT2 reduziu dor neuropática em indíviduos com neuralgia pós-herpética (RICE et al., 2014). Tantas evidencias sugerem que o RAT2 tem envolvimento com dor e inflamação e o torna um alvo terapêutico potencial para outros modelos, como o modelo de ataque agudo de gota.

Ainda assim, na literatura nunca foi verificada a contribuição dos receptores AT2 para o desenvolvimento de condições agudas como o ataque agudo de gota. Portanto, a hipótese do presente trabalho é que a ativação dos receptores AT2 no ambiente articular esteja relacionada com a iniciação do ataque agudo de gota. Neste contexto, propõem-se investigar o papel que o antagonismo dos receptores AT2 podem exercer sobre a dor e inflamação do ataque agudo de gota.

REFERÊNCIA BIBLIOGRÁFICA

BADER, M. Tissue renin–angiotensin–aldosterone systems: targets for pharmacological therapy. Annu. Rev. Pharmacol. Toxicol. 50, 439–465. 2010

BASBAUM AI, BAUTISTA DM, SCHERRER G, JULIUS D. Cellular and molecular mechanisms of pain. Cell. 139(2):267-284. 2009.

BENNETT, DL. & WOODS, CG. Painful and painless channelopathies. Lancet Neurol. 13, 587–599. 2014.

CAMPION EW, GLYNN RJ, DELABRY LO. Asymptomatic hyperuricemia. Risks and consequences in the Normative Aging Study. Am J Med. 82(3):421-6. 1987.

CAREY RM. Update on the role of the AT2 receptor. Curr Opin Nephrol Hypertens. 14(1):67-71. 2005.

CAREY RM. Newly discovered components and actions of the renin- angiotensin system. Hypertension. 62:818–822. 2013.

CHA SA, PARK BM, KIM SH. Angiotensin-(1-9) ameliorates pulmonary arterial hypertension via angiotensin type II receptor. Korean J Physiol Pharmacol. 22(4):447-456. 2018.

CHEN-XU M, YOKOSE C, RAI SK, PILLINGER MH, CHOI HK. Contemporary Prevalence of Gout and Hyperuricemia in the United States and Decadal Trends: The National Health and Nutrition Examination Survey, 2007-2016. Arthritis Rheumatol. 71(6):991-999. 2019.

CHOI HK, MOUNT DB, REGINATO AM. American College of Physicians, American Physiological Society, Pathogenesis of gout. Ann Intern Med. v. 143, p. 499-516. 2005.

CHOI HK. et al. Antihypertensive drugs and risk of incident gout among patients with hypertension: population-based case-control study. Br. Med. J. v. 12, p. 344: d8190. 2012.

COX JJ, REIMANN F, NICHOLAS AK, THORNTON G, ROBERTS E, SPRINGELL, K., et al. An SCN9A channelopathy causes congenital inability to experience pain. Nature 444, 894–898. 2006.

CHAKRABARTY A, LIAO Z, MU Y, SMITH PG. Inflammatory Renin-Angiotensin System Disruption Attenuates Sensory Hyperinnervation and Mechanical Hypersensitivity in a Rat Model of Provoked Vestibulodynia. J Pain. 19(3):264-277. 2018.

CUNHA TM, VERRI WA JR., VIVANCOS GG, MOREIRA IF, REIS S, PARADA CA, CUNHA FQ, FERREIRA SH. An electronic pressure-meter nociception paw

test for mice. Braz J Med Biol Res 37:401–407. 2004.

DALBETH N, CHOI HK, JOOSTEN LAB, et al. Gout. Nat Rev Dis Primers. 5(1):69. 2019.

DALBETH N, GOSLING AL, GAFFO A, ABHISHEK A. Gout [published correction appears in Lancet. 2021 May 15;397(10287):1808]. Lancet. 397(10287):1843-1855. 2021.

DANSER et al. The Angiotensin II Type 2 Receptor for Pain Control. Cell Press. v. 157, p. 1504-1506. 2014.

DAO VT et al. Nitric oxide up-regulates endothelial expression of angiotensin II type 2 receptors. Biochem Pharmacol. 112:24–36. 2016.

FEIG DI, KANG DH, JOHNSON RJ. Uric acid and cardiovascular risk. N. Engl. J. Med. v. 359, p. 1811–1821. 2008.

DEHLIN M, DRIVELEGKA P, SIGURDARDOTTIR V, et al. Incidence and prevalence of gout in Western Sweden. Arthritis Res Ther. 18:164. 2016.

DUBIN AE, PATAPOUTIAN A. NOCICEPTORS: the sensors of the pain pathway. J Clin Invest. 120(11):3760-3772. 2010.

GEORGE J, STRUTHERS AD. Role of urate, xanthine oxidase andthe effects of allopurinol in vascular oxidative stress. VascHealth Risk Manag. 5:265–72.4. 2009.

JOSHI VR. Rheumatology, Past, Present and Future. J Assoc Physicians India. 60 21-24. 2012.

JULIUS D, BASBAUM AI. Molecular mechanisms of nociception. Nature. 413(6852):203-210. 2001.

KAMBAYASHI Y, BARDHAN S, TAKAHASHI K, et al. Molecular cloning of a novel angiotensin II receptor isoform involved in phosphotyrosine phosphatase inhibition. J Biol Chem. 268(33):24543-24546. 1993.

KANDEL, ER.; SCHWARTZ, JH.; JESSEL, TM. Princípios de Neurociência. São Paulo: Manole. 2003.

KEENAN RT et al. (2011). Prevalence of contraindications and prescription of pharmacologic therapies for gout. Am J Med. 124(2):155-63. Feb. 2011.

KUTZING MK, FIRESTEIN BL. Altered uric acid levels and disease states. J Pharmacol Exp Ther. v. 324, p. 1–7. 2008.

KOBORI H, NANGAKU M, NAVAR LG, NISHIYAMA A. The intrarenal renin-angiotensin system: from physiology to the pathobiology of hypertension and kidney disease. Pharmacol Rev 59:251–287. 2007.

MATAVELLI LC, SIRAGY HM. AT2 receptor activities and pathophysiological implications. J Cardiovasc Pharmacol. 65(3):226-232. 2015.

MEYER, RA, RINGKAMP M, CAMPBELL JN, RAJA SN. Peripheral mechanisms of cutaneous nociception.MILLAN, MJ. Descending control of pain. Prog Neurobiol 66(6): 355–474. 2006.

MOGIL, JS. Animal models of pain: progress and challenges. Nat. Rev. Neurosci. 10, 283–294. 2009.

MURALIDHARAN A, WYSE BD, SMITH MT. (2014). Analgesic Efficacy and Mode of Action of a Selective Small Molecule Angiotensin II Type 2 Receptor Antagonist in a Rat Model of Prostate Cancer-Induced Bone Pain. Pain Med. 15(1):93-110. 2014.

National Center for Biotechnology Information (2022). PubChem sumário de composto para CID 5311345. Acessado em 22 de janeiro de 2022. Disponível em https://pubchem.ncbi.nlm.nih.gov/compound/pd123319.

National Center for Biotechnology Information (2022). PubChem sumário de gene para o gene 186, AGTR2 - angiotensin II receptor type 2 (human). Acessado em 22 de janeiro de 2022. Disponível em https://pubchem.ncbi.nlm.nih.gov/gene/AGTR2/human.

NEOGI, T. Clinical practice. Gout. N Engl J Med, v. 364, p. 443-452. 2011.

PASSOS-SILVA DG, BRANDAN E, SANTOS RA. Angiotensins as therapeutic targets beyond heart disease. Trends Pharmacol Sci. 36(5):310-20. 2015.

PUEYO ME, MICHEL JB. Angiotensin II receptors in endothelial cells. Gen. Pharmacol. 29:691–696. 1997.

RAI SK, AVIÑA-ZUBIETA JA, MCCORMICK N, et al. The rising prevalence and incidence of gout in British Columbia, Canada: Population-based trends from 2000 to 2012. Semin Arthritis Rheum. 46(4):451-456. 2017.

RANJIT A, KHAJEHPOUR S, AGHAZADEH-HABASHI A. Update on Angiotensin II Subtype 2 Receptor: Focus on Peptide and Nonpeptide Agonists. Mol Pharmacol. 99(6):469-487. 2021.

RAJA SN, CARR DB, COHEN M, et al. The revised International Association for the Study of Pain definition of pain: concepts, challenges, and compromises. Pain. 161(9):1976-1982. 2020.

REES et al. Optimizing current treatment of gout. Nature Reviews Rheumatology. 10, 271–283. 2014.

RICE ASC et al. EMA401, an orally administered highly selective angiotensin II type 2 receptor antagonist, as a novel treatment for postherpetic neuralgia: a randomised, double-blind, placebo-controlled phase 2 clinical trial. Lancet; 383(9929):1637-47. 2014

RICHETTE P, BARDIN T. Gout. Lancet; 375:318-28. 2010.

SCHLESINGER N. Treatment of acute gout. Rheum Dis ClinNorth Am. 40:329–41. 2014

SCHLESINGER N. The safety of treatment options available for gout. Expert Opin Drug Saf. 16(4):429-436. 2017.

SHERRINGTON CS. The Integrative Action of the Nervous System. Cambridge, UK: Cambridge Univ. Press. 1906.

SILVA CR, OLIVEIRA SM, HOFFMEISTER C, et al. The role of kinin B_1 receptor and the effect of angiotensin I-converting enzyme inhibition on acute gout attacks in rodents. Annals of the Rheumatic Diseases .75:260-268. 2016.

SMITH, MAREE & MURALIDHARAN, ARJUN. Targeting angiotensin II type 2 receptor pathways to treat neuropathic pain and inflammatory pain. Expert Opinion on Therapeutic Targets. 18(12). 2014.

SO AK, MARTINON F. Inflammation in gout: mechanisms and therapeutic targets. Nat Rev Rheumatol. 13(11):639-647. 2017.

TAYLOR WJ, FRANSEN J, JANSEN TL, et al. Study for Updated Gout Classification Criteria: Identification of Features to Classify Gout. Arthritis Care Res (Hoboken). 267(9):1304-1315. 2015.

TERKELTAUB R, BUSHINSKY DA, BECKER MA. Recent developments in our understanding of the renal basis of hyperuricemia and the development of novel antihyperuricemic therapeutics. Arthritis Res Ther. v. 8 Suppl1:S4. 2006.

TERENZI R, MANETTI M, ROSA I. Angiotensin II type 2 receptor (AT2R) as a novel modulator of inflammation in rheumatoid arthritis synovium. Sci Rep 7. 2017.

TORRES R, MACDONALD L, CROLL SD, et al. Hyperalgesia, synovitis and multiple biomarkers of inflammation are suppressed by interleukin 1 inhibition in a novel animal model of gouty arthritis. Ann Rheum Dis. 68:1602-8. 2009.

VARGAS RA, VARELA MILLÁN JM, FAJARDO BONILLA E. Renin-angiotensin system: Basic and clinical aspects-A general perspective. Endocrinol diabetes y Nutr 69:52–62. 2022.

VON BANCHET GS, RICHTER J, HÜCKEL M, ROSE C, BRÄUER R, SCHAIBLEHG. Fibroblast-like synovial cells from normal and inflamed knee joints differently affect the expression of painrelated receptors in sensory neurones: a co-culture study. Arthritis Res Ther; 9(1):R6. 2007.

WOOLF CJ. What is this thing called pain? J Clin Invest, v. 120, p. 3742-3744. 2010.

XU J, CARRETERO OA, ZHU L, SHESELY EG, RHALEB NE, DAI X, WANG L, YANG JJ, YANG XP. Protective role of AT(2) and B(1) receptors in kinin B(2)-receptor-knockout mice with myocardial infarction. Clin Sci (Lond). 124(2):87-96. 2013.

YOKOSE C, MCCORMICK N, CHOI HK. The role of diet in hyperuricemia and gout. Curr Opin Rheumatol. 33(2):135-144. 2021.

ZHU Y, PANDYA BJ, CHOI HK. Comorbidities of gout and hyperuricemia in the US general population: NHANES 2007-2008. Am J Med. 125(7):679-687. 2012.

CAPÍTULO II
ARTIGO

Angiotensin type 2 receptor antagonism as a new target to manage acute gout arthritis

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ABSTRACT

Objective: The present study aimed to evaluate the analgesic and anti-inflammatory potential of the angiotensin type 2 receptor (AT_2R) antagonism in a mouse model of acute gout attack.

Methods: Male wild type (WT) C57BL/6 mice treated with the AT_2R antagonist, PD123319 (10 pmol/joint), or Agtr2[tm1a(EUCOMM)Wtsi] mice, received intraarticular (IA) injection of MSU (100 µg/joint), and were analyzed for mechanical allodynia, thermal hyperalgesia, spontaneous nociception and ankle edema development several times after injections. Additionally, WT mice IA cotreated with PD123319 received the IA administration of angiotensin II (0.05–5 nmol/joint) and were also evaluated for pain and edema development. Following, ankle joint tissue samples from WT mice IA treated with PD123319 plus MSU crystals were assessed for myeloperoxidase activity, IL-1β release, mRNA expression analyses and nitrite/nitrate levels, 4 h after injections. All treatments were compared to MSU only or vehicle IA injected mice.

Results: AT$_2$R antagonism has robust antinociceptive effects on mechanical allodynia (44% prevention) and spontaneous nociception (56% prevention), as well as anti-inflammatory effects preventing edema formation (11%), reducing myeloperoxidase activity (54% prevention) and IL-1β levels (30% prevention). Additionally, Agtr2^{tm1a} mutant mice have largely reduced painful signs of gout. Angiotensin II administration causes pain and inflammation, which was prevented by AT$_2$R antagonism. PD123319 treatment also reduces NO concentrations (70% prevention) and AT$_2$R mRNA levels in comparison with MSU untreated mice.

Conclusion: Our findings suggest that AT$_2$R activation contributes to the acute gout attack in experimental mouse model. Therefore, the antagonism of AT$_2$R may be a potential therapeutic option to manage gout arthritis.

1. INTRODUCTION

Gouty arthritis is characterized by hyperuricemia (serum urate levels ≥ 7 mg/L) leading the formation and deposition of monosodium urate (MSU) crystals in the joints, resulting in disabling pain, being the actual most common cause of inflammatory arthritis worldwide (Dalbeth et al. 2018, 2021). However, for the growing number of individuals with gout, current therapeutic options remain limited and are largely contraindicated, mainly because of the concomitant presence of comorbidities that these individuals exhibit which reduce therapeutic efficacy, increase toxicity and provide drug interactions (Schlesinger 2017; Elfishawi et al. 2018).

Hypertension is among the most frequently comorbidities associated with gout and some of the drugs used to treat this symptom, such as angiotensin converting enzyme inhibitors (ACEi) have been shown to increase risk of developing an acute gout attack

(Choi et al. 2012; Zhu et al. 2012; Elfishawi et al. 2018). It is well know that inhibition of angiotensin converting enzyme (ACE) can result in the regulation of the kinin and the renin-angiotensin systems and, our group have previously demonstrated that kinin system is only partially involved in the acute gout attack, including those precipitated by the use of ACEi (Silva et al. 2016). However, there are no studies until now evaluating the possible involvement of renin-angiotensin system in gout. So, we hypothesized that a dysregulation on the renin-angiotensin system may also respond for pain and inflammation observed in gout.

Angiotensin system has two major G protein-coupled receptor subtypes, the angiotensin II type 1 receptor (AT_1R) and the angiotensin II type 2 receptor (AT_2R) (Vargas et al. 2022). The AT_2R can be expressed in different cell types present in the articular environment, such peripheral sensory neurons, endothelial cells, chondrocytes, synoviocytes and peripheral macrophages (Pueyo and Michel 1997; Kawakami et al. 2012a; Terenzi et al. 2017; Shepherd et al. 2018a). Recent findings demonstrate that the involvement of the angiotensin system targeting AT_2R for pain sensitization and, AT_2R antagonism has antinociceptive effects in animal models of neuropathic, inflammatory and bone cancer pain (Smith et al. 2013; Muralidharan et al. 2014; Chakrabarty et al. 2018; Shepherd et al. 2018a, b). In addition, a phase II clinical trial demonstrate that AT_2R inhibition reduced neuropathic pain in individuals with post-herpetic neuralgia, supporting efficacy and safety for human treatment (Rice et al. 2014). Despite the growing interest, is still unclear the role of AT_2R for gout arthritis development.

Thus, we proposed to investigate if the activation of AT2R was important for the acute gout attack, accounting for pain and inflammation using a acute gout mice model triggered by intra-articular MSU crystals injection. We also analyzed the possible

mechanisms involved in the process, confirming the therapeutic potential of AT2R antagonism for the management of gout.

2. METHODS

2.1 Animals

All animal handling and experimental procedures were approved by the Ethics Committee in Animal Experimentation of the Federal University of Uberlândia (CEUA/UFU-080/16) or by the Animal Welfare Ethical Review Board (AWERB) of King's College London (for experiments in KCL). Adult male C57BL/6/J/UFU mice (20-25 g, bred in house) provided by UFU REBIR (UFU rodent animal breeding group), and C57BL/6N wild-type (WT) strain isogenic compared to Agtr2^{tm1a} mutant mice provided by KCL BSU (biological services unit), were used in the experiments. Agtr2$^{tm1a(EUCOMM)Wtsi}$ (Agtr2^{tm1a}) mutant mice were generated at Wellcome Trust Sanger Institute on a C57BL/6N genetic background (Skarnes et al. 2011; White et al. 2013). These mice carry a promoter-driven knockout-first allele, with a large cassette inserted in the intron before the targeted critical exon 3 which interferes with transcription leading to knockout of AT$_2$R expression. Further details can be found at www.mousephenotype.org.

Animals were kept in a controlled-temperature environment in individual ventilated cages (5 per cage), with wood shaving bedding and nesting material, maintained at 22±1°C, with a 12 hours light/dark cycle (lights on at 7:00 am) and fed with rodent chow (Puro Lab 22 PB pelleted form, Global Diet 2018, Harlan, Lombardia for mice) and tap water *ad libitum*. Animals were normally allowed to acclimatize to their experimental room for 1 hour before experiments. Behavioral observations were

performed in a blind fashion by investigators and followed the Animal Research Reporting In vivo Experiments (ARRIVE) guidelines as well as in accordance with the Home Office (UK) regulations and the Animals (Scientific Procedures) Act 1986. The treatments were only done in anesthetized mice (isoflurane 2%, 100% O_2 1L/min). The number of mice used in each experiment are presented in graph legend, being used the total of 183 mice for the study.

2.2 Reagents and Drugs

Otherwise indicated, all reagents were from Sigma (Sigma, St Louis, MO, USA) and dissolved using phosphate buffered saline (PBS) as vehicle. AT_2R antagonist, PD123319 ditrifluoroacetate, was purchased from TOCRIS Bioscience, USA ("1361" batch no: 3A/189254). MSU crystals were prepared as according to Hoffmeister et al. (2011). Polarized light microscopic examination confirmed that the crystals were rod-shaped and varied in length (12 ± 2 μm). Therefore, the crystals were aliquoted (100 μg) and keep stored for use only once to avoid contamination. All intra-articular injections had a final volume of 10 μl.

2.3 MSU-induced acute gout attack animal model and treatments

The acute gout attack animal model was induced by an intra articular (IA) injection of MSU crystals (10–100 μg/joint) administered on the tibio tarsal articulation (ankle joint) of the animals (Silva et al. 2016; Rossato et al. 2020). PBS was used as the vehicle.

The AT_2R antagonist, PD123319, (10 pmol/joint) was co-administered by an intra articular injection with MSU crystals or Angiotensin II, or orally administrated (1 mg/kg) 30 min before MSU crystals IA injections (Muralidharan et al. 2014; Shepherd et al.

2018a). Angiotensin II was also administered alone (0.05–5 nmol/joint) by IA route (Shepherd et al. 2018a, with some modifications). After the injections the animals were analyzed for nociception and inflammation development at, several time points (1, 2, 4, 6 and 24 hours).

2.4 Mechanical allodynia

The mechanical allodynia was measured in mice using von Frey hair filaments of increasing strength (0.008–1.4 g), applied in the center of the hind paw with a gentle stimulus until the filaments bend for 4 seconds. The measurements followed the "Up and Down" method as described by Chaplan et al. (1994). Briefly, mice were placed in an acrylic cage individually (9 x 7 x 11 cm) with a wire grid floor, 1 hour before start of behavioral testing. When the animal had no exploratory movements, defecation and was not resting the stimulus began. Firstly, the filament of intermediate intensity (0.4 g) was used. When the animal responded to the stimulus, a lower filament was used (0.16 g). If the animal does not respond to the stimulus, the next larger filament was used (0.6 g). This procedure was repeated until the weakest filament able to elicit a response was identified and was considered to be the mechanical nociceptive threshold, expressed in mg (log) (Cunha et al. 2004).

2.5 Spontaneous Nociception

To measure spontaneous nociception in C57BL/6/J/UFU mice, the animals were placed in an acrylic cage as previously described and observed according to their behavior to support the weight of the body on the paw corresponding to the injected joint on a scale of spontaneous nociception, without stimulus (Coderre and Wall 1987; Silva et al. 2016). The evaluation of spontaneous pain of the animals was performed considering three-point

scale where: 0 was considered when the paw pressure was normal, with equal weight distribution on both hind-paws; score 1 was considered when the hind paw was slightly raised but still touching the grid; score 2 was considered when paw pressure was moderately reduced, with foot curled with only some parts of the foot lightly touching the floor; and finally score 3 was considered when the paw pressure was severely reduced, with the paw completely elevated within the body.

2.6 Cold thermal sensitivity

The thermal sensitivity was evaluated against a cold stimulus using acetone (50 µl) that was sprinkled topically with the aid of a syringe to the center of the plantar surface of the hind paw (Caspani et al. 2009) with modifications. The animals were placed in an acrylic cage as previously described. The nociceptive response time (paw licking or paw shaking) of animals was timed for 2 minutes and expressed in seconds.

2.7 Edema

As an inflammatory parameter we evaluated edema formation of the mice ankle joint articulation using a plethysmometer (Ugo Basile, Monvalle, Italy). The values were expressed in milliliters of water dislocated by the articulation and compared with the baseline measure or control groups.

2.8 Myeloperoxidase (MPO) activity and IL-1β measurement

To evaluate the inflammatory neutrophil infiltration, we analyzed MPO activity and IL-1β levels. Only for this analysis we performed knees joint MSU (100 ug/joint) injection due the final volume necessary to the assays. Following, 2 h after MSU injections, the injected joint (knee) synovial cavity was washed three times with 5 µL and diluted to a final volume of 50 µl of PBS to obtain the synovial lavage sample (Pinto et

al. 2010; Rossato et al. 2020). Vehicle injected mice were used as control. The samples were centrifuged for at 800 g for 8 minutes at 4 °C, the pellet was collected and resuspended in 50 μL of PBS-EDTA for MPO assay. The supernatant was collected and diluted in 20 μL of PBS-EDTA for IL-1β levels determination.

For MPO activity assessment, the resuspended pellet was homogenized in 80 mM NaPO$_4$ buffer (pH 5.4) containing 0.5% hexadecyltrimethylammonium bromide (HTAB) and evaluated by colorimetric assay based on peroxidation of tetramethylbenzidine (TMB). The reaction was stopped by adding 4M H$_2$SO$_4$ and determined by spectrophotometry (Spectra Max-250; Molecular Devices, Sunnyvale, CA, USA) at 450 nm. Results were presented as the number of neutrophils $\times$ 10^4/mg of joint (Alves-Filho et al. 2010).

IL-1β was measured by ELISA following the manufacturer's instructions (R&D Systems, Minneapolis, MN, USA). Results were expressed as picograms of cytokine per milligram of synovial fluid.

2.9 Nitric oxide (NO) concentration measurement

To evaluate the NO$_2$ and NO$_3$ articular tissue concentration, 4 h after PD123319, (10 pmol/joint) plus MSU injections, or MSU alone, synovial lavage was obtained as previously described (Pinto et al. 2010). Samples were prepared as according to (Miranda et al. 2001; Rossato et al. 2020). Briefly 100 μl of standard nitrate solution (range 2mM–0.125mM) was serially diluted at 96-well plates, the collected samples were prepared with 100 μl of Griess reagent plus 40 μl of vanadium chloride (0.02mg/mL), then incubated for 1 hour at 37 ºC. Measurements were made using a spectrophotometer with wavelength absorption (540 nm), and results were expressed in μM concentration.

2.10 RNA isolation and qPCR

For PCR analyzes, mice joint samples were collected 4 hours after administration of MSU. The tissue was held in 500 µl of TRIzol reagent (Sigma-Aldrich, St. Louis, MO) and stored at -90 ºC, until the day of the experiment, then the samples were homogenized with a Polytron Homogenizer (Thermo Scientific, USA). Manufacturer's instructions were followed. Quantity and purity of isolated RNA were checked by a NanoDrop spectrophotometer (Thermo Scientific, USA) with wavelength absorption ratio (260/280 nm) and 500 ng of RNA was transcribed into cDNA using reverse transcription reaction (Superscript II; Invitrogen Life Technologies). qPCR reactions have the final volume of 13 µl with 6.25 µl of PowerUp SYBR Green Master Mix (Applied Biosystems), 0.5 µl forward primer, 0.5 µl reverse primer, 4.75 µl Milli-Q water (Millipore Corporation) and 1 µl sample. Reactions were performed in 96-well plates compatibles with the Axygen Scientific Real-Time PCR System. Following initial denaturation, samples were cycled through denaturation (95 ºC, 10 s), annealing (60 ºC, 60 s) and extension (60 ºC, 60 s) for 40 cycles, followed by melt curve analysis to ascertain specificity of amplification. Primers used (table 1).

2.11 Statistical Analyses

Kolmogorov-Smirnov normality test was used to determine whether the data values had normal distributions. Differences among 3 or more groups at one point were analyzed by one-way analysis of variance (ANOVA) followed by Newman-Keuls or Dunnett's posttest. Differences among 3 or more groups at different times were analyzed by two-way ANOVA followed by Bonferroni's posttest. Statistical analysis was performed using GraphPad Software 5.0 (GraphPad Software, San Diego, CA, USA). P values less than 0.05 were considered significant. To meet the ANOVA assumptions, the

mechanical hyperalgesia data were log transformed prior to statistical analysis.

3. RESULTS

3.1 AT$_2$R activation is involved in MSU-triggered acute gout attack nociception.

The MSU-induced acute gout attack model in mice was confirmed by the decreased paw withdrawal thresholds in response to mechanical stimulus, spontaneous and thermal nociception development, when compared to the vehicle group (supplementary Fig. 1). The doses of 30 and 100 μg of MSU crystals evoked a significant nociceptive mechanical and spontaneous response, and the 100 μg dose was selected for following experiments (Rossato et al. 2020). Interestingly the intraarticular (IA). coadministration of the AT$_2$R antagonist, PD123319 (10 pmol/joint) plus MSU crystals (100 μg/joint), prevented mechanical allodynia in 4-6 h (Fig. 1A), spontaneous nociception from 2-4 h (Fig. 1B), and cold thermal nociceptive responses (Fig. 1C) from 1-24 h after IA injections. The same nociceptive parameters were analyzed for mice treated with AT$_2$R antagonist (PD123319, 1 mg/kg) given orally half hour before MSU IA injection and shown prevention both of mechanical allodynia and spontaneous nociception 4-6 h after the injection (supplementary figure Fig. 2). Following the study, we used the IA regimen of treatment once it allows the evaluation of AT$_2$R involvement in acute gout attacks without any loss to the analyses and conclusions and also implies lower quantities of the antagonist.

To confirm the previous data, we induced the acute gout attack model in the Agtr2^{tm1a} mutant mice, which is effectively deficient for the AT2 receptor. As expected, we noticed that IA injection of MSU (100 μg) in control group (C57BL/6N mice) induced

a significant reduction in the paw mechanical withdrawal threshold when compared to the PBS IA injection group (Fig. 1D). In agreement with the data obtained with PD123319 treatment, we also observed that the Agtr2^{tm1a} mutant mice did not develop mechanical allodynia during MSU-triggered acute gout attack.

Moreover, it was already been demonstrated that AT$_2$R can be expressed by macrophages (Shepherd et al. 2018b). Accordingly, we observed that peripheral macrophage depletion by administration of liposome-encapsulated clodronate leads to an antinociceptive response, as previously observed (supplementary Fig. 3A) (Rossato et al. 2020). The depletion of macrophages cells was confirmed by a viability test (supplementary Fig. 3B).

3.2 AT$_2$R activation is involved in MSU-triggered acute gout attack inflammation.

As expected, additionally to pain, we confirmed that MSU-triggered acute gout attack model, was also able to induce an inflammatory process characterized by articular edema, neutrophil migration and increased IL-1β production. Curiously, the edema was prevented by the local treatment with PD123319 (10 pmol/joint) (11±1,1 % of prevention) (Fig. 2A), and the MSU-induced myeloperoxidase activity was also decreased in the PD123319-treated group in comparison with control group (54,6±4,4 % of prevention) (Fig. 2B). Moreover, PD123319 also prevented the IL-1β production (release) (30,6±9,8 % of prevention) (Fig. 2C).

3.3 Angiotensin II induced nociception is prevented by AT$_2$R antagonism.

To assess a specific action of AT$_2$R on articulation, we treated the mice with the AT$_2$R agonist, angiotensin II, and evaluated the development of mechanical allodynia. We noticed that the agonist injection induced mechanical allodynia at all tested doses

starting 2 h after the injection (Fig. 3A). The 0.5 nmol dose was chosen to be used for following experiments. AT_2R antagonist, PD123319 (10 pmol/joint) was able to prevent the mechanical allodynia induced by angiotensin II (Fig. 3B). Angiotensin II was also able to induce spontaneous nociception and cold thermal nociceptive responses from 1-4 h, which was prevented by IA coadministration of the AT_2R antagonist, PD123319 (10 pmol/joint) (Fig. 3C and D).

3.4 Nitric oxide involved in MSU-induced acute gout attack is decreased by AT2R antagonism

The AT_2R activation can result in an increased synthesis and release of NO, which is involved in rodent and human acute gout attack (Carey et al. 2001; Dao et al. 2016; Gumanova et al. 2017; Rossato et al. 2020). In agreement, the NO_2 and NO_3 concentration at articular synovial fluid of MSU injected group is highly increased compared to the vehicle injected group. The co-administration with the AT_2R antagonist, PD123319 (10 pmol/joint), was able to significantly prevent the increase in NO concentration (70 ± 1 % of prevention) (Fig. 4A).

3.5 AT$_1$R, AT$_2$R, and ACE2 mRNA levels are altered in the ankle joint after MSU injection and AT2R antagonism

We found an increase in AT_1R mRNA levels in the acute gout attack model suggesting increased expression of the AT_1R when MSU was intra-articular administered, which was decreased when AT_2R was antagonized with PD123319 (Fig. 5A). Despite of the slight altered mRNA levels of AT_2R and ACE2, they were both not statistically significant when comparing vehicle to MSU group (Fig. 5B and D). However, when MSU plus PD123319-treated group was compared to MSU group, the mRNA levels of AT_2R

and ACE2 decreased significantly in both groups (Fig 5B and D). The results of the ACE1 qPCR shows no difference between the groups (Fig. 5C).

4. DISCUSSION

Gout is an inflammatory arthritis, characterized by the deposition of MSU crystals in the joints, resulting in disabling and excruciating painful acute episodes (Dalbeth et al. 2019, 2021). Recent studies indicate that the angiotensin system is involved in pain sensitization, however, this has not yet been observed for pain and inflammation in acute gout attack (Shepherd et al. 2018a, b). Here, we demonstrate that antagonism of type 2 angiotensin receptors prevented the acute attack of gout, alleviating pain and inflammation. The disease has a large presence of comorbidities, which contributes to low adherence to conventional treatments. In addition, hypertension is highly prevalent, affecting about 74% of individuals with gout (Zhu et al. 2012; Elfishawi et al. 2018; Dehlin et al. 2020). Therefore, there is an increased risk of developing acute attacks of gout after the use of some antihypertensive drugs, such as angiotensin converting enzyme inhibitors (Choi et al. 2012). Thus, our discovery that AT2R as a therapeutic target may contribute to better management of gout.

Pain in gout is clinically described as intense and disabling, where individuals often affected by this pathological condition have several problems in performing basic functions, such as walking (Busso and So 2010; Taylor et al. 2015; Dalbeth et al. 2019). Furthermore, spontaneous pain, and joint allodynia are the main characteristics of gouty pain, which strongly affects the patients' quality of life, causing numerous public health, economic, and social problems. In our study we explored the ankle MSU crystals injection animal model of gout because of the accuracy of the nociceptive measurement, due to the behavioral evaluation of the primary site of inflammation, as previously discussed

(Rossato et al. 2020). In accordance, we confirmed pain development after MSU injections and importantly demonstrated that the AT_2R antagonism has antinociceptive effects in a gout mouse model. Also, we demonstrated for the first time that AT2R genetic depletion can prevent from MSU inducing mechanical allodynia, suggesting an important role of angiotensin system on acute gout arthritis attack pain context. Similarly, the AT_2R pharmacological blockade has been described previously as a strategy to prevent neuropathic, inflammatory and bone cancer pain in other animal models (Smith et al. 2013, 2016; Muralidharan et al. 2014; Shepherd et al. 2018b).

Besides pain, we also observed prevention of inflammation development after MSU injections by the AT_2R antagonism. The MSU crystals injection reproduces on rodents the inflammatory characteristics observed in gout patients, such as redness, articular edema, neutrophil migration, as well as increased levels of IL-1β (Dalbeth et al. 2019). It is important to observe that neutrophils are the main cells present on gout synovial fluid in humans and that IL-1β is the key cytokine driving the inflammatory process of the acute gout attack (Mitroulis et al. 2013; Dumusc and So 2015; So and Martinon 2017). Interestingly, we observe that AT_2R antagonism was able to partially reduce articular edema, neutrophil infiltration and IL-1β release. These findings suggest that AT_2R antagonism has a potential to treat acute gout attacks and may be also efficient to be used on the treatment of different acute inflammatory diseases.

Although AT_2R has recently described to play a role in pain sensitization, some discrepant results are still observed about its expression in nociceptive neurons and literature recently suggested that angiotensin II does not directly influence sensory neuron's function (Shepherd et al. 2018a). Moreover, there are just few studies demonstrating the AT_2R expression in articular tissues (Kawakami et al. 2012b;

Tsukamoto et al. 2013; Kawahata et al. 2015). Then, we decided to analyze what effects would have angiotensin II injection on naive mice ankle and observed pain development, which was prevented by the AT2R antagonist treatment. This exciting findings from our investigation points out that the angiotensin system is important player for the articular pain development, more specifically, the activation of the AT2R, since PD123319 was able to prevent pain development induced by angiotensin II. Likewise, regarding the angiotensin system, it is worth noting that angiotensin I can be converted in angiotensin II, via ACE activity (Vargas et al. 2022). It was previously shown that MSU can increase ACE activity, and also induce an increase in angiotensin II formation, which would be able to activate the AT2R in the articular microenvironment (Silva et al. 2016). However, the molecular mechanisms of these events remain to be elucidated.

Interestingly, Shepherd (Shepherd et al. 2018a) demonstrated a crosstalk between peripheral macrophages and sensory neurons, mediated by AT_2R via TRPA1 redox signaling, as critical for peripheral pain sensitization. Macrophages are also present in the articular microenvironment and are involved in MSU-induced nociception and inflammation (Rossato et al. 2020). Depletion of articular resident macrophages has been shown to result in decreased cytokine production, including IL-1β, the central cytokine of gout inflammation (Martin et al. 2009; Rossato et al. 2020).

We have recently demonstrated and reproduced in our group that macrophage depletion using the clodronate liposome approach is able to prevent MSU-induced gout pain in mice (Rossato et al. 2020). Additionally, as suggested for peripheral pain sensitization, the TRPA1 redox signaling has been previously described as critical for gout pain and inflammation development (Trevisan et al. 2014). Altogether, these findings also indicate that AT_2R expression in macrophages is an important receptor

related to articular pain and inflammation, such as observed in gout arthritis.

Besides TRPA1 involvement in gout, TRPV1 channels are also described as important for gout pain and inflammation (Hoffmeister et al. 2011; Rossato et al. 2020). More specifically, we have recently demonstrated that increased levels of nitric oxide, triggered by TLR4 expressed in phagocytic cells, results in TPRV1 activation and 1L-1β release during acute gout attack (Rossato et al. 2020). Nitric oxide, as well as the enzyme responsible for its production, the inducible nitric oxide synthase, were already described as present in synovial fluid of gouty patients and MSU-stimulated cell culture (Glair et al. 1996; Chen et al. 2004). Interestingly, AT_2R are positively regulated by NO in endothelial cells, and its activation leads to an increased synthesis and release of NO (Carey et al. 2001; Dao et al. 2016). Corroborating, here we demonstrated that AT2R antagonism was able to reduce NO release in the synovial fluid in response to MSU stimulation in vivo. As we recently observed, articular macrophage cells stimulated with MSU can release NO and this process is related to TRP channels activation and IL-1B release (Rossato et al. 2020). AT2R may interact with TRP channels for IL-1β release in the articular scenario. However, more studies are necessary to elucidate these events. Taken together, our findings suggest that pain and inflammation in MSU-induced gout attack seems to be dependent of AT2 expression and NO release by macrophage, indicating the AT_2R as a new and important target to improve gout management.

Furthermore, we also investigated by quantitative PCR the mRNA levels of AT_1R, AT_2R, ACE1, and ACE2. We observed that MSU intra-articular injection only significantly increased AT_1R mRNA, even an increase in AT_2R mRNA can be observed, it was not significant. On the other hand, AT_2R antagonism was able to reduce AT_1R, AT_2R and ACE2 mRNA significantly when compared to MSU-induced gout arthritis.

These results confirm the complex regulation of the angiotensin system, where each receptor and enzyme can counteract to the expression/activity regulation of the others (AbdAlla et al. 2001; Kostenis et al. 2005). Forte et al. (2016) demonstrated that AT_1R antagonist treatment increased the antinociceptive effects of angiotensin 1-7 via Mas receptor activation, suggesting that ACE2-Ang (1-7) / Mas receptor axis may opposite to the activity of AT_1R signaling, which was also demonstrated by Nemoto et al. (2014). In gout arthritis, these observations reinforce that MSU can activate the angiotensin system, where ACE2 activity was increased after MSU intra-articular injection (Silva et al., 2016).

In summary, we demonstrated the important role of AT2R for gout arthritis pain and inflammation, indicating that AT2R involvement in gout includes NO and IL-1B release signaling. The mechanisms described in the present study that accounts for the AT2R contribution for the development of acute gout attacks are depicted in Fig. 6. Altogether, our results suggest that AT2R can be explored as therapeutic targets to improve the management of gout acute attacks. Altogether, our results suggest that AT_2R are involved in gout arthritis pain and inflammation and can be explored as therapeutic targets to improve management of gout acute attacks.

5. ACKNOWLEDGMENT

6. REFERENCES

AbdAlla S, Lother H, Abdel-tawab AM, Quitterer U (2001) The Angiotensin II AT2 Receptor Is an AT1 Receptor Antagonist. J Biol Chem 276:39721–39726. https://doi.org/10.1074/jbc.M105253200

Alves-Filho JC, Snego F, Souto FO, et al (2010) Interleukin-33 attenuates sepsis by enhancing neutrophil influx to the site of infection. Nat Med 16:708–712. https://doi.org/10.1038/NM.2156

Busso N, So A (2010) Mechanisms of inflammation in gout. Arthritis Res. Ther. 12

Carey RM, Jin XH, Siragy HM (2001) Role of the angiotensin AT2 receptor in blood pressure regulation and therapeutic implications. In: American Journal of Hypertension. Elsevier Inc.

Caspani O, Zurborg S, Labuz D, Heppenstall PA (2009) The contribution of TRPM8 and TRPA1 channels to cold allodynia and neuropathic pain. PLoS One 4:.

https://doi.org/10.1371/journal.pone.0007383

Chakrabarty A, Liao Z, Mu Y, Smith PG (2018) Inflammatory Renin-Angiotensin System Disruption Attenuates Sensory Hyperinnervation and Mechanical Hypersensitivity in a Rat Model of Provoked Vestibulodynia. J Pain 19:264–277. https://doi.org/10.1016/j.jpain.2017.10.006

Chaplan SR, Bach FW, Pogrel JW, et al (1994) Quantitative assessment of tactile allodynia in the rat paw. J Neurosci Methods 53:55–63. https://doi.org/10.1016/0165-0270(94)90144-9

Chen L, Hsieh MS, Ho HC, et al (2004) Stimulation of inducible nitric oxide synthase by monosodium urate crystals in macrophages and expression of iNOS in gouty arthritis. Nitric Oxide - Biol Chem 11:228–236. https://doi.org/10.1016/j.niox.2004.09.003

Choi HK, Soriano LC, Zhang Y, Garciá Rodriǵuez LA (2012) Antihypertensive drugs and risk of incident gout among patients with hypertension: Population based case-control study. BMJ 344:. https://doi.org/10.1136/bmj.d8190

Coderre TJ, Wall PD (1987) Ankle joint urate arthritis (AJUA) in rats: an alternative animal model of arthritis to that produced by Freund's adjuvant. Pain 28:379–393. https://doi.org/10.1016/0304-3959(87)90072-8

Cunha TM, Verri WA, Vivancos GG, et al (2004) An electronic pressure-meter nociception paw test for mice. Brazilian J Med Biol Res 37:401–407. https://doi.org/10.1590/S0100-879X2004000300018

Dalbeth N, Choi HK, Joosten LAB, et al (2019) Gout. Nat. Rev. Dis. Prim. 5

Dalbeth N, Gosling AL, Gaffo A, Abhishek A (2021) Gout. Lancet 397:1843–1855. https://doi.org/10.1016/S0140-6736(21)00569-9

Dalbeth N, Phipps-Green A, Frampton C, et al (2018) Relationship between serum urate concentration and clinically evident incident gout: An individual participant data analysis. Ann Rheum Dis 77:1048–1052. https://doi.org/10.1136/annrheumdis-2017-212288

Dao VTV, Medini S, Bisha M, et al (2016) Nitric oxide up-regulates endothelial expression of angiotensin II type 2 receptors. Biochem Pharmacol 112:24–36. https://doi.org/10.1016/j.bcp.2016.05.011

Dehlin M, Jacobsson L, Roddy E (2020) Global epidemiology of gout: prevalence, incidence, treatment patterns and risk factors. Nat Rev Rheumatol 16:380–390. https://doi.org/10.1038/s41584-020-0441-1

Dumusc A, So A (2015) Interleukin-1 as a therapeutic target in gout. Curr. Opin. Rheumatol. 27:156–163

Elfishawi MM, Zleik N, Kvrgic Z, et al (2018) The rising incidence of gout and the increasing burden of comorbidities: A population-based study over 20 years. J Rheumatol 45:574–579. https://doi.org/10.3899/jrheum.170806

Forte BL, Slosky LM, Zhang H, et al (2016) Angiotensin-(1-7)/Mas receptor as an antinociceptive agent in cancer-induced bone pain. Pain 157:2709–2721. https://doi.org/10.1097/j.pain.0000000000000690

Glair EWS, Wilkinson WE, Lang T, et al (1996) Increased expression of blood mononuclear cell nitric oxide synthase type 2 in rheumatoid arthritis patients. J Exp Med 184:1173–1178. https://doi.org/10.1084/jem.184.3.1173

Gumanova NG, Deev AD, Klimushina M V., et al (2017) Serum nitrate and nitrite are associated with the prevalence of various chronic diseases except cancer. Int Angiol 36:160–166. https://doi.org/10.23736/S0392-9590.16.03674-9

Hoffmeister C, Trevisan G, Rossato MF, et al (2011) Role of TRPV1 in nociception and edema induced by monosodium urate crystals in rats. Pain 152:1777–1788. https://doi.org/10.1016/j.pain.2011.03.025

Kawahata H, Sotobayashi D, Aoki M, et al (2015) Continuous infusion of angiotensin II modulates hypertrophic differentiation and apoptosis of chondrocytes in cartilage formation in a fracture model mouse. Hypertens Res 38:382–393. https://doi.org/10.1038/hr.2015.18

Kawakami Y, Matsuo K, Murata M, et al (2012a) Expression of Angiotensin II Receptor-1 in Human Articular Chondrocytes. Artic ID 2012:. https://doi.org/10.1155/2012/648537

Kawakami Y, Matsuo K, Murata M, et al (2012b) Expression of Angiotensin II Receptor-1 in Human Articular Chondrocytes. Arthritis 2012:1–7. https://doi.org/10.1155/2012/648537

Kostenis E, Milligan G, Christopoulos A, et al (2005) G-protein-coupled receptor Mas is a physiological antagonist of the angiotensin II type 1 receptor. Circulation 111:1806–1813. https://doi.org/10.1161/01.CIR.0000160867.23556.7D

Martin WJ, Walton M, Harper J (2009) Resident macrophages initiating and driving inflammation in a monosodium urate monohydrate crystal-induced murine peritoneal model of acute gout. Arthritis Rheum 60:281–289. https://doi.org/10.1002/art.24185

Miranda KM, Espey MG, Wink DA (2001) A rapid, simple spectrophotometric method for simultaneous detection of nitrate and nitrite. Nitric Oxide - Biol Chem 5:62–71. https://doi.org/10.1006/niox.2000.0319

Mitroulis I, Kambas K, Ritis K (2013) Neutrophils, IL-1β, and gout: Is there a link?

Semin. Immunopathol. 35:501–512

Muralidharan A, Wyse BD, Smith MT (2014) Analgesic efficacy and mode of action of a selective small molecule angiotensin II type 2 receptor antagonist in a rat model of prostate cancer-induced bone pain. Pain Med (United States) 15:93–110. https://doi.org/10.1111/pme.12258

Nemoto W, Ogata Y, Nakagawasai O, et al (2014) Angiotensin (1-7) prevents angiotensin II-induced nociceptive behaviour via inhibition of p38 MAPK phosphorylation mediated through spinal Mas receptors in mice. Eur J Pain (United Kingdom) 18:1471–1479. https://doi.org/10.1002/ejp.512

Pinto LG, Cunha TM, Vieira SM, et al (2010) IL-17 mediates articular hypernociception in antigen-induced arthritis in mice. Pain 148:247–256. https://doi.org/10.1016/j.pain.2009.11.006

Pueyo ME, Michel JB (1997) Angiotensin II receptors in endothelial cells. Gen. Pharmacol. 29:691–696

Rice ASC, Dworkin RH, McCarthy TD, et al (2014) EMA401, an orally administered highly selective angiotensin II type 2 receptor antagonist, as a novel treatment for postherpetic neuralgia: A randomised, double-blind, placebo-controlled phase 2 clinical trial. Lancet 383:1637–1647. https://doi.org/10.1016/S0140-6736(13)62337-5

Rooijen N Van, Sanders A (1994) Liposome mediated depletion of macrophages: mechanism of action, preparation of liposomes and applications. J Immunol Methods 174:83–93. https://doi.org/10.1016/0022-1759(94)90012-4

Rossato MF, Hoffmeister C, Trevisan G, et al (2020) Monosodium urate crystal interleukin-1β release is dependent on Toll-like receptor 4 and transient receptor

potential V1 activation. Rheumatol (United Kingdom) 59:233–242. https://doi.org/10.1093/rheumatology/kez259

Schlesinger N (2017) The safety of treatment options available for gout. Expert Opin Drug Saf 16:429–436. https://doi.org/10.1080/14740338.2017.1284199

Schmidt-Weber CB, Rittig M, Buchner E, et al (1996) Apoptotic cell death in activated monocytes following incorporation of clodronate-liposomes. J Leukoc Biol 60:230–244. https://doi.org/10.1002/jlb.60.2.230

Shepherd AJ, Copits BA, Mickle AD, et al (2018a) Angiotensin II triggers peripheral macrophage-to-sensory neuron redox crosstalk to elicit pain. J Neurosci 38:7032–7057. https://doi.org/10.1523/JNEUROSCI.3542-17.2018

Shepherd AJ, Mickle AD, Golden JP, et al (2018b) Macrophage angiotensin II type 2 receptor triggers neuropathic pain. Proc Natl Acad Sci U S A 115:E8057–E8066. https://doi.org/10.1073/pnas.1721815115

Silva CR, Oliveira SM, Hoffmeister C, et al (2016) The role of kinin B1 receptor and the effect of angiotensin I-converting enzyme inhibition on acute gout attacks in rodents. Ann Rheum Dis. https://doi.org/10.1136/annrheumdis-2014-205739

Skarnes WC, Rosen B, West AP, et al (2011) A conditional knockout resource for the genome-wide study of mouse gene function. Nature 474:337–344. https://doi.org/10.1038/nature10163

Smith MT, Anand P, Rice ASC (2016) Selective small molecule angiotensin II type 2 receptor antagonists for neuropathic pain: Preclinical and clinical studies. In: Pain. Lippincott Williams and Wilkins, pp S33–S41

Smith MT, Woodruff TM, Wyse BD, et al (2013) A Small Molecule Angiotensin II Type 2 Receptor (AT2R) Antagonist Produces Analgesia in a Rat Model of Neuropathic

Pain by Inhibition of p38 Mitogen-Activated Protein Kinase (MAPK) and p44/p42 MAPK Activation in the Dorsal Root Ganglia. Pain Med (United States) 14:1557–1568. https://doi.org/10.1111/pme.12157

So AK, Martinon F (2017) Inflammation in gout: Mechanisms and therapeutic targets. Nat. Rev. Rheumatol. 13:639–647

Taylor WJ, Fransen J, Jansen TL, et al (2015) Study for updated gout classification criteria: Identification of features to classify gout. Arthritis Care Res 67:1304–1315. https://doi.org/10.1002/acr.22585

Terenzi R, Manetti M, Rosa I, et al (2017) Angiotensin II type 2 receptor (AT2R) as a novel modulator of inflammation in rheumatoid arthritis synovium. Sci Rep 7:. https://doi.org/10.1038/s41598-017-13746-w

Trevisan G, Hoffmeister C, Rossato MF, et al (2014) TRPA1 receptor stimulation by hydrogen peroxide is critical to trigger hyperalgesia and inflammation in a model of acute gout. Free Radic Biol Med 72:200–209. https://doi.org/10.1016/j.freeradbiomed.2014.04.021

Tsukamoto I, Inoue S, Teramura T, et al (2013) Activating types 1 and 2 angiotensin II receptors modulate the hypertrophic differentiation of chondrocytes. FEBS Open Bio 3:279–284. https://doi.org/10.1016/J.FOB.2013.07.001

Vargas Vargas RA, Varela Millán JM, Fajardo Bonilla E (2022) Renin-angiotensin system: Basic and clinical aspects-A general perspective. Endocrinol diabetes y Nutr 69:52–62. https://doi.org/10.1016/J.ENDINU.2021.05.012

White JK, Gerdin AK, Karp NA, et al (2013) XGenome-wide generation and systematic phenotyping of knockout mice reveals new roles for many genes. Cell 154:452. https://doi.org/10.1016/j.cell.2013.06.022

Zhu Y, Pandya BJ, Choi HK (2012) Comorbidities of gout and hyperuricemia in the US general population: NHANES 2007-2008. Am J Med 125:. https://doi.org/10.1016/j.amjmed.2011.09.033

FIGURE LEGENDS

Figure 1. Prevention of MSU-induced nociceptive response mediated by treatment with angiotensin II receptor type 2 selective antagonist PD123319 and MSU-induced gout attack model on Agtr2[tm1a] mutant mice. (A and D) Mechanical allodynia, (B) spontaneous nociception, (C) thermal nociceptive responses. N = 6 mice per group. Each column represents the mean ± SEM. # $P<0.05$ and ## $P<0.01$ and ### $P<0.001$ represent significant differences compared to vehicle group. * $P<0.05$, ** $P<0.01$ and *** $P<0.001$ represent significant differences compared to MSU injected group. The statistical analysis was performed using two-way ANOVA followed by Bonferroni's post-test in (A) and one-way ANOVA followed by Dunnet's post-test in each interval (B), (C), (D).

Figure 2. Prevention of MSU-induced inflammation, cell migration and inflammatory mediators' production mediated by treatment with angiotensin II receptor type 2 selective antagonist PD123319. (A) Articular edema, (B) Myeloperoxidase activity and (C) IL-1β levels. N = 5 (A and B) and 10 (C) mice per group. Each column represents the mean ± SEM. ### $P<0.001$ represent significant differences compared to vehicle group. * $P<0.05$ and ** $P<0.01$ represent significant differences compared to MSU injected group. The statistical analysis was performed using one-way ANOVA followed by Dunnet's post-test.

Figure 3. Nociceptive responses induced by angiotensin II and prevention of angiotensin

II-induced nociceptive response caused by angiotensin II receptor type 2 selective antagonist, PD123319. (A and B) Mechanical allodynia, (C) spontaneous nociception and (D) cold thermal nociceptive responses. N = 6 mice per group. Angiotensin II (Angio II). Each column represents the mean ± SEM. # P<0.05 and ## P<0.01 and ### P<0.001 represent significant differences compared to vehicle group. * P<0.05, ** P<0.01 and *** P<0.001 represent significant differences compared to Angiotensin II injected group. The statistical analysis was performed using two-way ANOVA followed by Bonferroni's test (A) and (B) and one-way ANOVA followed by Dunnet's post-test in each interval (C) and (D).

Figure 4. Angiotensin II receptor antagonist, PD123319, decreases NO levels in synovial fluid of mice submitted to acute gout attack.

N = 6 mice per group. Each column represents the mean ± SEM. ### P<0.001 represent significant differences compared to vehicle group. *** P<0.001 represent significant differences compared to MSU injected group. The statistical analysis was performed using one-way ANOVA followed by Dunnet's post-test.

Figure 5. Angiotensin II receptor antagonist, PD123319 attenuates the mRNA levels of AT_2R, AT_1R and ACE2 in the acute gout attack model, which is increased when compared to the group injected into the vehicle. (A) AT_1R mRNA levels, (B) AT_2R mRNA levels, (C) ACE1 mRNA levels and (D) ACE2 RNA levels. N = 6-9 mice per group. Each column represents the mean ± SEM. ## P<0.01 represent significant differences compared to vehicle group. * P<0.05 represent significant differences compared to MSU injected group. The statistical analysis was performed using one-way ANOVA followed by Dunnet's post-test.

Figure 6. Mechanisms described in the present study that accounts for the AT2R contribution for the development of acute gout attacks.

TABLES AND FIGURES

Table 1

Name of primer	Primer sequence for 5' 3'
AT$_1$R-F	GGCCAGTGTTTTTCTTTTGAATTTAGCAC
AT$_1$R-R	TGAACAATAGCCAGGTATCGATCAATGC
AT$_2$R-F	CTGCTGGGATTGCCTTAATG
AT$_2$R-R	CATCTTCAGGACTTGGTCAC
ACE-F	CACTATGGGTCCGAGTACAT
ACE-R	ATCATAGATGTTGGACCAGG
ACE2-F	GTGCACAAAGGTGACAATGG
ACE2-R	ATGCGGGGTCACAGTATGTT
GAPDH-F	GGGTGTGAACCACGAGAAAT
GAPDH-R	CCACAGTCTTCTGAGTGGCA

Figure 1
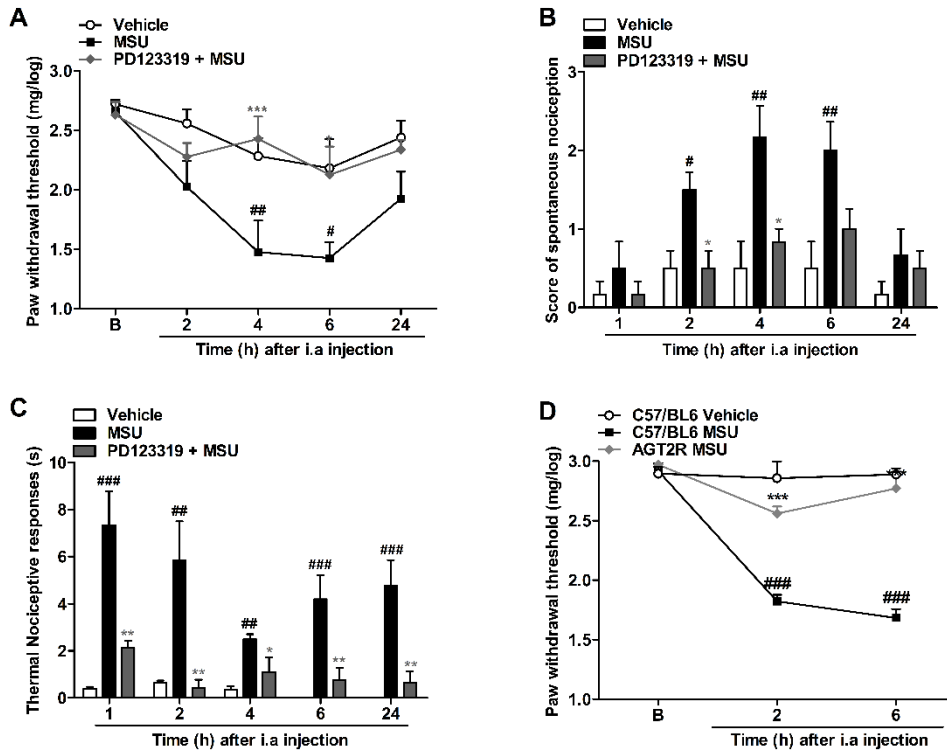
Figure 2
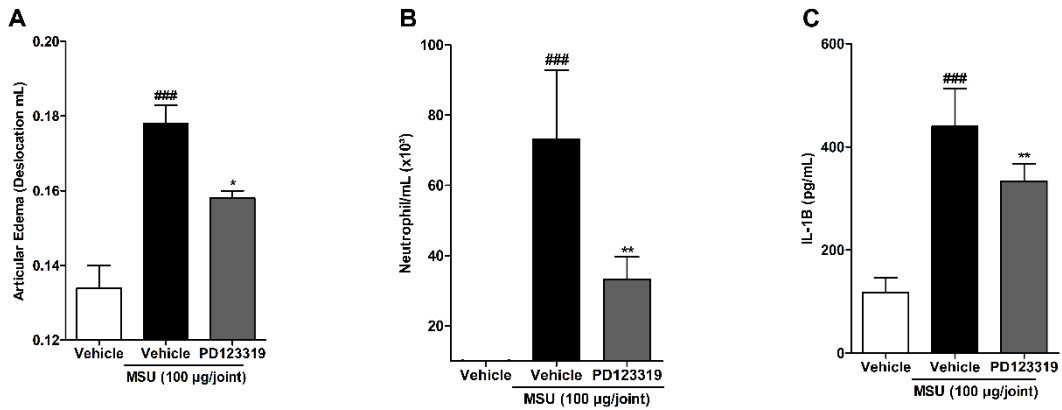
Figure 3
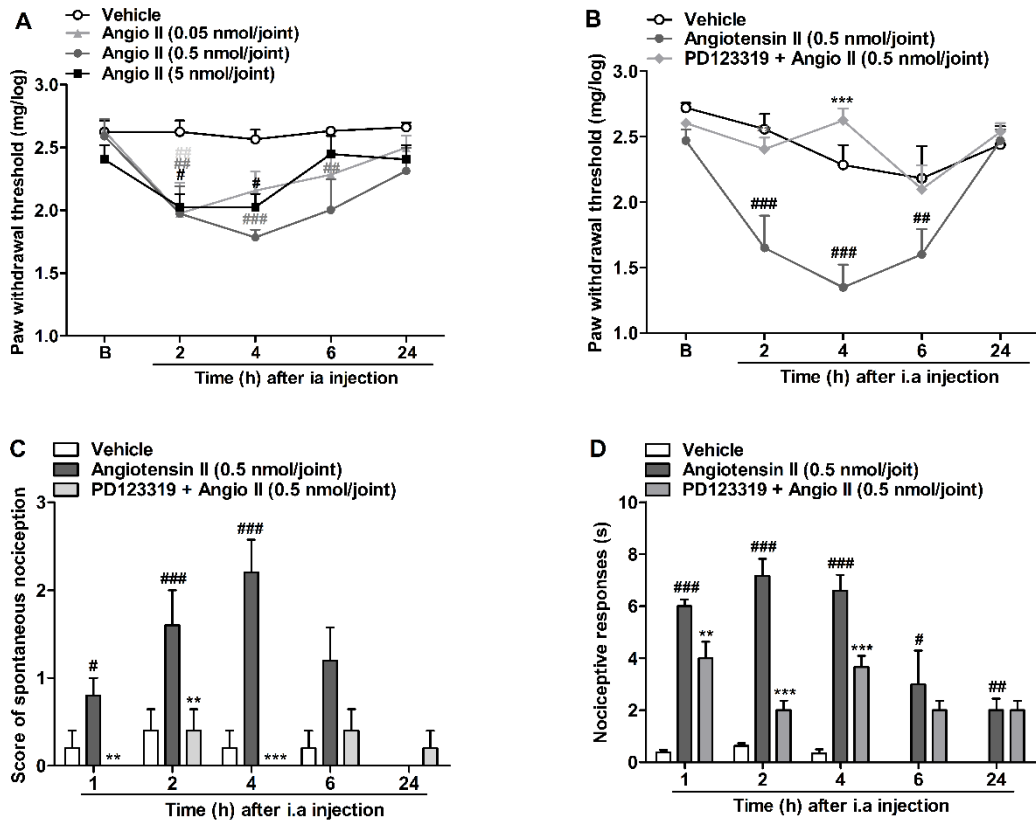
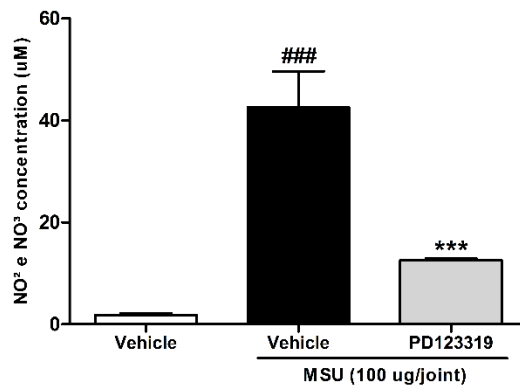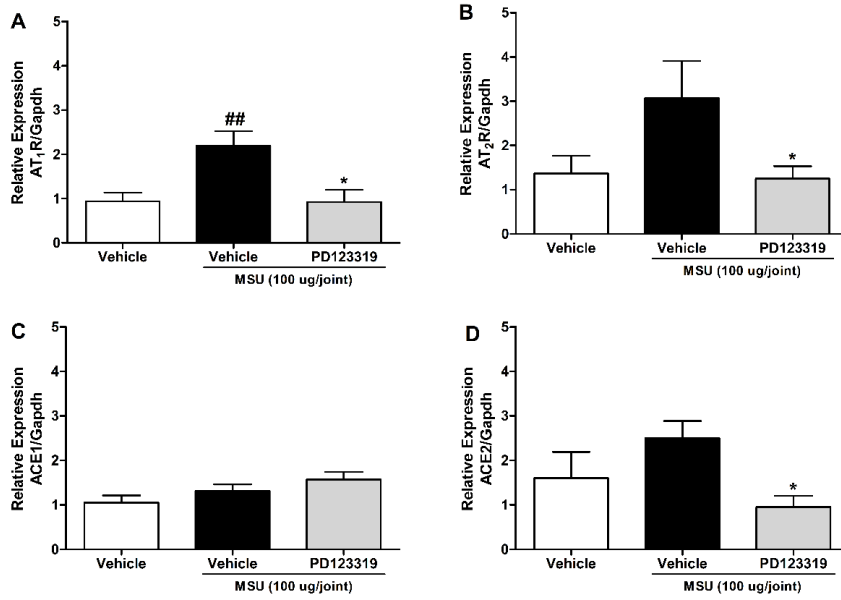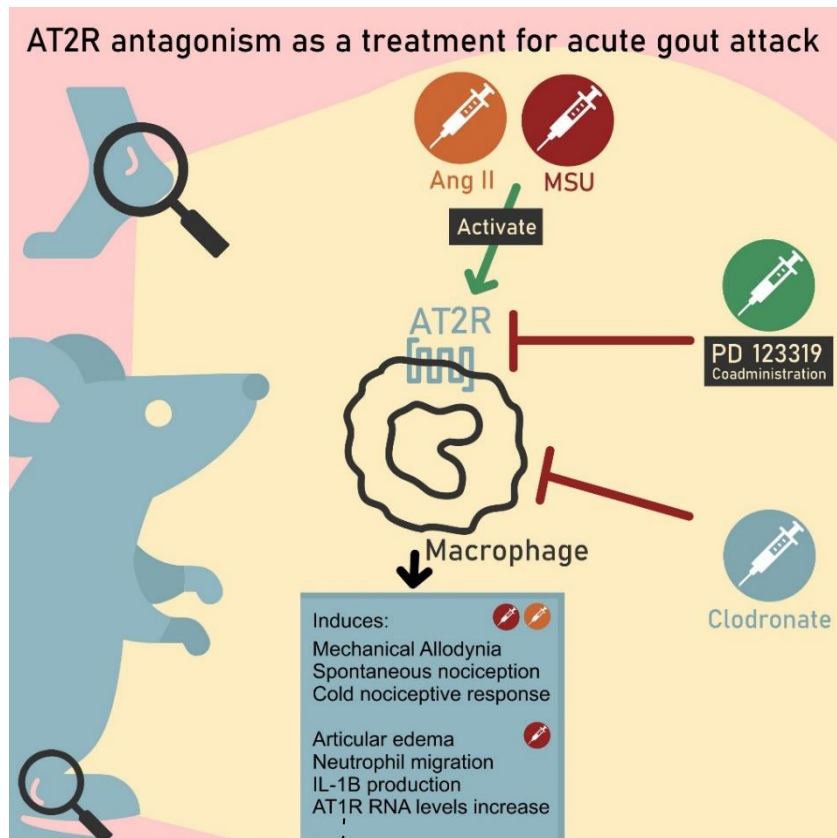**Figure 4**

Figure 5

Figure 6

SUPPLEMENTARY MATERIAL

Supplementary Methods

Clodronate macrophage depletion

Multilamellar liposomes encapsulating the drug clodronate: dichloromethylene diphosphonate (CL2MPD) were prepared according (Rooijen and Sanders 1994). The liposomes are provided in a partner with professor Jair Pereira da Cunha Junior from ICBIM UFU. Briefly 86 mg phosphatidylcholine and 8 mg cholesterol are dissolved in chloroform in a round bottom flask. By vacuum rotary evaporation at 37 °C, a thin film formed on the wall of the flask. Clodronate (2.5 g), dissolved in 10 ml of PBS and enclosed by gentle shaking for 10 min was kept at room temperature for two hours and sonicated at 20 °C for three minutes. After an additional two hours at room temperature, free clodronate was removed by washing three times with PBS (20 000 g, 10 min).

Sixteen μl of a clodronate laden liposome suspension (containing 30 μg CL2MDP) was injected once in the ankle articulation joint of the mice, seven days before the acute attack model. As control, PBS containing liposomes were injected. After the depletion of macrophages, the animals were submitted to the acute gout attack, then analyzed by nociceptive and inflammatory parameters several times until 24 h after the injection.

Generation of bone marrow-derived macrophages (BMDM) and MTT viability assay

BMDM were used to validate the biological activity of liposome-encapsulated clodronate. Briefly, femur bones from C57BL/6/J/UFU mice (6-8 weeks old) were dissected and, in sterile culture hood, a 23-gauge needle and syringe filled with RPMI-1640 medium were used to flush bone marrow out; RPMI-1640 was previously

supplemented with antibiotic (100 U/ mL penicillin and 100 µg/ mL streptomycin), L-glutamine (2 mM) and fetal bovine serum (10%). Bone marrow cells were seeded (5-8 x 10^6 cells) in not treated polystyrene Petri dishes (Corning) and incubated in 10 mL of RPMI-1640 which were also supplemented with 30% of L-929 cell conditioned medium (LCCM) which was used as supplier of macrophage-colony stimulating factor (m-CSF); cells were maintained at 37ºC and 5% of CO_2 atmosphere. Following tree day 10 mL of fresh medium in the same conditions were added to cell culture and, 7-8 later adherent macrophage were harvest.

Macrophages were seeded to 96 wells plate at density of 0.2 x 10^6 cells/ well and were incubated (37ºC and 5% of CO_2) overnight to allow cell adhesion. Next, macrophages were incubated with different concentrations of liposome-encapsulated clodronate and cell culture was maintained during additional 48 h. To cell viability analysis we used MTT assay. For this, supernatant was removed and MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) (Sigma-Aldrich) at 5 mg/ mL and solubilized in RPMI-1640 were added to cells which were incubated for 2 h. The purple formazan formed during metabolism of live cells was solubilized in dimethyl sulfoxide (DMSO). The absorbance was determined at 570 nm in microplate reader (Versa Max, Molecular Device). Cell viability was calculated by comparing to control group which was considered as 100% of viability.

Phagocytic synovial lining cells ingest the clodronate-liposomes. During digestion the membrane of the liposome is disrupted, the clodronate sets free, and the cell dies of apoptosis (Schmidt-Weber et al. 1996). If injected in ankle articulation joints of control C57BL/6 mice, optimal depletion of synovial lining cells was observed seven days after injection

Supplementary Figures and Figure Legends

## MSU-induced acute gout attack
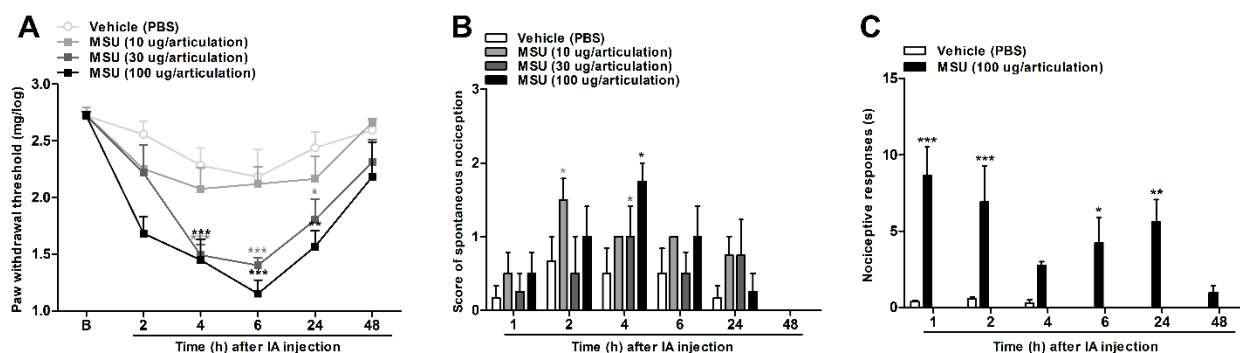
Supplementary Figure 1. Nociceptive responses elicited by different concentrations of monosodium urate crystals intra articular injection. (A) Mechanical allodynia, (B) spontaneous nociception and (C) thermal nociceptive responses. N=6 per group. Phosphate buffer saline (PBS), monosodium urate crystals (MSU). Each column represents the mean ± SEM. * $P<0.05$, ** $P<0.01$ and *** $P<0.001$ represent significant differences compared to vehicle injected group. The statistical analysis was performed using two-way ANOVA followed by Bonferroni's test (A) and one-way ANOVA followed by Dunnet's post-test in each interval (B) and (C).

Antagonist AT2 oral treatment for MSU-triggered acute gout attack
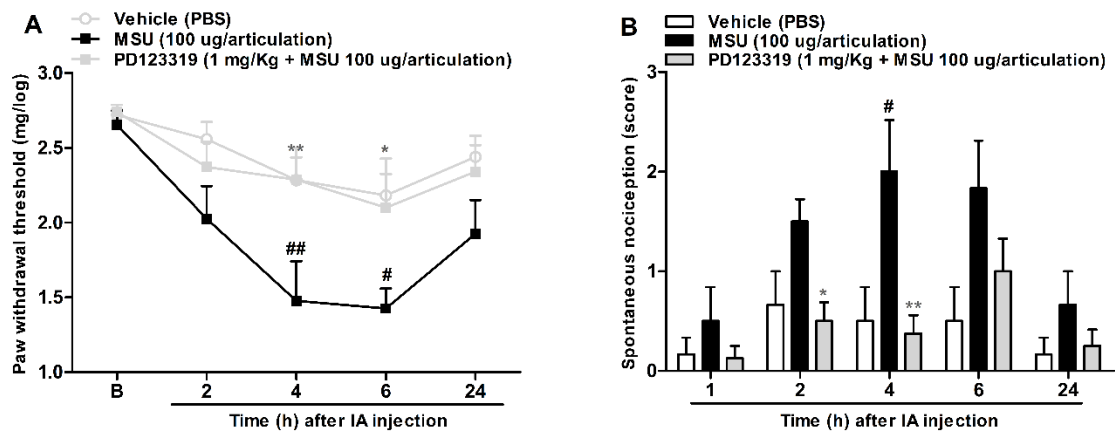
Supplementary Figure 2. Prevention of nociceptive response caused by monosodium urate crystals with angiotensin II receptor type 2 selective antagonist PD123319 oral route 30 minutes before intra articular injection of the crystals. (A) Mechanical allodynia and (B) spontaneous nociception. N=6 per group. Phosphate buffer saline (PBS), monosodium urate crystals (MSU). Each column represents the mean ± SEM. # $P<0.05$ and ## $P<0.01$ represent significant differences compared to vehicle group. * $P<0.05$ and ** $P<0.01$ represent significant differences compared to MSU injected group. The statistical analysis was performed using two-way ANOVA followed by Bonferroni's test (A) and one-way ANOVA followed by Dunnet's post-test in each interval (B).

MSU-triggered acute gout attack involve peripheral macrophage AT2R
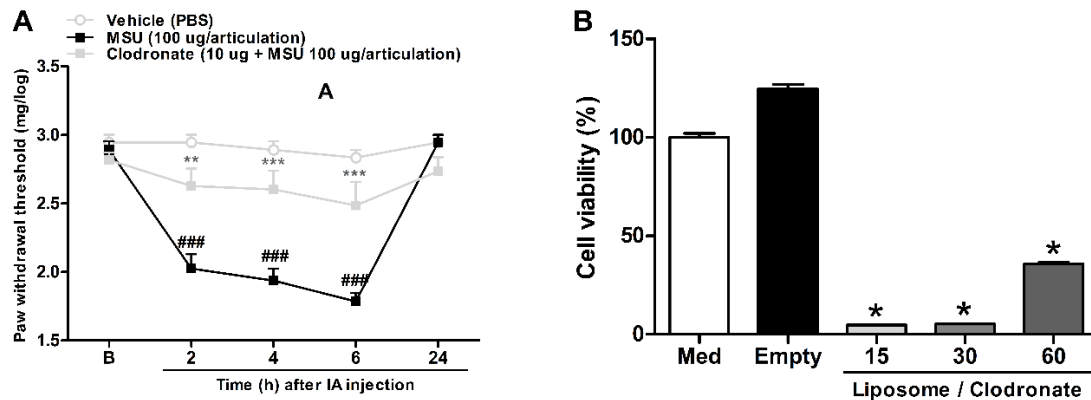
Supplementary Figure 3. Antinociceptive effects of liposomes filled with clodronate. (A) Mechanical allodynia, (B) Clodronate Viability. N = 6 per group. Phosphate buffer saline (PBS). Each column represents the mean ± SEM. ### P<0.001 represent significant differences compared to vehicle group. (A) Each column represents the mean ± SEM. ** P<0.01 and *** P<0.001 represent significant differences compared to MSU injected group or in (B) empty group. (A) The statistical analysis was performed using two-way ANOVA followed by Bonferroni's post-test.

Supplementary References

Van Rooijen N, Sanders A. Liposome mediated depletion of macrophages: mechanism of action, preparation of liposomes and applications. J Immunol Methods 1994;174:83– 93.

Schmidt-Weber CB, Rittig M, Buchner E, et al. Apoptotic cell death in activated monocytes following incorporation of clodronate-liposomes. J Leukoc Biol 1996;60(2):230-244.