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EFEITOS ANTI-NOCICEPTIVO E ANTI-EDEMATOGÊNICO DA GLIBENCLAMIDA EM UM MODELO DE GOTA AGUDA EM RATOS

TESE DE DOUTORADO

Rosane Maria Souza dos Santos

Santa Maria, RS, Brasil,

2013

EFEITOS ANTI-NOCICEPTIVO E ANTI-EDEMATOGÊNICO DA GLIBENCLAMIDA EM UM MODELO DE GOTA AGUDA EM RATOS

Por

Rosane Maria Souza dos Santos

Tese apresentada no 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 **Doutor em Bioquímica Toxicológica**.

Orientador: Dr. Jamil Assreuy

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Santa Maria, RS, Brasil

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Universidade Federal de Santa Maria
Centro de Ciências Naturais e Exatas
Programa de Pós-Graduação em Ciências Biológicas:
Bioquímica Toxicológica

A comissão examinadora, abaixo assinada,
aprova a Tese de Doutorado

EFEITOS ANTI-NOCICEPTIVO E ANTI-EDEMATOGÊNICO DA GLIBENCLAMIDA EM UM MODELO DE GOTA AGUDA EM RATOS

elaborada por

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como requisito parcial para obtenção do grau de

Doutor em Bioquímica Toxicológica

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Santa Maria, 23 de fevereiro de 2013

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"... A mudança está nas tuas mãos.

Reprograma a tua meta,

Busca o bem e viverás melhor.

Embora ninguém possa voltar atrás

e fazer um novo começo,

qualquer um pode começar agora

e fazer um novo fim."

Chico Xavier

RESUMO

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

EFEITOS ANTI-NOCICEPTIVO E ANTI-EDEMATOGÊNICO DA GLIBENCLAMIDA EM UM MODELO DE GOTA AGUDA EM RATOS

AUTORA: Rosane Maria Souza dos Santos
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LOCAL E DATA DA DEFESA: Santa Maria, 23 de fevereiro de 2013

A gota é uma forma de artrite inflamatória, causada pela precipitação de cristais de urato monossódico (MSU) nas articulações. A forma aguda de gota está associada a episódios inflamatórios súbitos e dolorosos caracterizados por uma grande infiltração de neutrófilos. Apesar dos anos de estudo sobre a gota, o seu tratamento ainda é um desafio pela relativa ineficácia dos fármacos disponíveis no mercado. Assim, a busca por novos agentes terapêuticos mais efetivos e seguros se faz necessário. Desta forma, o objetivo deste estudo foi investigar o possível potencial farmacológico da glibenclamida em um modelo de gota aguda induzida por MSU em ratos. Os cristais de MSU produziram nocicepção e edema quando injetados na articulação do tornozelo de ratos. O tratamento com glibenclamida (3 mg/kg, s.c.) ou dexametasona (8 mg/kg, s.c., usada como controle positivo) reduziu a nocicepção espontânea (67 ± 11% e 70 ± 7% de inibição, respectivamente) e o edema (28 ± 7% e 77 ± 7% de inibição, respectivamente) induzidos pelo MSU, 6 horas após a injeção do cristal. O número de leucócitos infiltrados no líquido sinovial, assim como a liberação de interleucina 1β (IL-1β) e de prostaglandina E_2 (PGE_2) foram consideravelmente aumentados, 6 horas após a injeção de MSU na articulação, porém esses efeitos não foram revertidos pelo tratamento com glibenclamida (3 mg/kg, s.c.). Em contrapartida, dexametasona reduziu a infiltração de leucócitos e a liberação de IL-1β e de PGE_2. Para confirmar se a dose utilizada de glibenclamida foi capaz de bloquear os canais de K_{ATP}, foi avaliado os níveis de glicose no sangue dos animais. A glibenclamida reduziu (23 ± 2%) e a dexametasona aumentou a glicemia dos ratos quando comparado aos animais tratados com veículo /MSU. Assim, frente aos efeitos desempenhados pela glibenclamida sobre a nocicepção e edema induzidos pelo MSU, sugere-se que esta sulfonilureia possa ser uma opção interessante como um tratamento adjuvante na dor observada em ataques agudos de gota.

Palavras-Chave: Glibenclamida; Nocicepção; Edema; Leucócito; Inflamassoma; Prostaglandina E_2; Canal de potássio sensível a ATP; Interleucina 1β.

ABSTRACT

PhD Thesis
Graduate Course in Biological Sciences: Toxicological Biochemistry
Universidade Federal de Santa Maria, RS, Brazil

ANTI-NOCICEPTIVE AND ANTI-EDEMATOGENIC EFFECTS OF GLIBENCLAMIDE IN AN ACUTE MODEL OF GOUT ARTHRITIS IN RATS

AUTHOR: Rosane Maria Souza dos Santos
ADVISOR: Jamil Assreuy
CO-ADVISOR: Juliano Ferreira
PLACE AND DATE OF THE PUBLIC DEFENSE: Santa Maria, February 23, 2013.

Gout is one form of inflammatory arthritis, which is caused by the precipitation of crystals of monosodium urate (MSU) in the joints. Acute gout is associated with sudden and painful inflammatory episodes characterized by high neutrophil infiltration. In spite of years of study gout treatment remains a challenge due to its relative ineficcacy. Thus, search for new and efficient therapies is necessary. The objective of this study was to investigate the involvement of glibenclamide in a model of acute gout in rats induced by MSU. MSU crystals produced nociception and edema when injected into the ankle joint of rats. Treatment with glibenclamide (3 mg/kg, s.c.) or dexamethasone (8 mg/kg, s.c., used as a positive control) decreased spontaneous nociception (67% ± 11 and 70 ± 7% inhibition, respectively) and edema (28 ± 7% and 77 ± 7% inhibition, respectively) induced 6 hours after MSU injection. The number of leukocyte infiltrates in the synovial fluid as well as the release of interleukin 1β (IL-1β) and prostaglandin E_2 (PGE_2) significantly increased at 6 hours after injection of MSU joint, but these effects were not reversed by treatment with glibenclamide (3 mg/kg, s.c.). In contrast, dexamethasone reduced the leukocyte infiltration and release of IL-1β and PGE_2. To confirm if the dose of glibenclamide was able to block the K_{ATP} channels, we determined the levels of glucose in the blood of animals. Glibenclamide decreased (23 ± 2%) and dexamethasone increased the blood glucose of the rats compared to vehicle-treated animals / MSU. Therefoe, the effects of glibenclamide on nociception and edema induced MSU, suggests that this sulfonylurea may be an interesting option as an adjunct therapy in pain observed in acute attacks of gout.

Keywords: Glibenclamide; Nociception; Edema; Leukocyte; Inflamassoma; Prostaglandin E_2; ATP-sensitive potassium channel; Interleukin 1β.

LISTA DE ILUSTRAÇÕES

Tese

Manuscrito

LISTA DE ABREVIATURAS E SIGLAS

ATP	Adenosina Trifosfato
AINES	Anti-inflamatórios não esteroidais
ANTI-IL	Anticorpos contra interleucina
ANOVA	Análise de variancia
DMSO	Dimetilsulfóxido
DMEM	Dulbecco's Modified Eagle Medium
ELISA	Enzimaimunoensaio
FDA	Food and Drug Administration
IL-1β	Interleucina 1 beta
IL-1	Interleucina 1
IL-8	Interleucina 8
IL-18	Interleucina 18
K^+	Potássio
K_{ATP}	Potássio sensível ao ATP
KC	Canal de potássio
MSU	Urato monossódico
NHANES III	Third National Health and Nutrition Examination Survey
NSAIDS	Nonsteroidal anti-inflammatory drugs
NF-kB	Fator de transcrição nuclear kappa B
PBS	Tampão fosfato salina
PGE_2	Prostaglandina E_2
SEM	Erro padrão
SUR	Receptor de sulfonilureias
α- TNF	Fator de Necrose Tumoral alfa

LISTA DE APÊNDICES

SUMÁRIO

APRESENTAÇÃO

Nos itens **INTRODUÇÃO** e **REVISÃO BIBLIOGRÁFICA** 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 Manuscrito, o qual se encontra no item **RESULTADOS**. As seções Materiais e Métodos, Resultados, Discussão e Referências Bibliográficas encontram-se no próprio manuscrito e representam a íntegra deste estudo.

O item **CONCLUSÃO** encontrado no final desta tese, apresentam interpretações e comentários gerais sobre o Manuscrito contido neste trabalho.

As **REFERÊNCIAS BIBLIOGRÁFICAS** referem-se somente às citações que aparecem nos itens **INTRODUÇÃO** e **REVISÃO BIBLIOGRÁFICA** desta tese.

No item **APÊNDICE** é encontrado um Manuscrito em preparação referente à outra produção, como primeiro autor, realizado durante o período de Doutoramento da aluna.

1. INTRODUÇÃO

A gota é uma doença antiga que tem fascinado e afetado praticamente todas as civilizações ao longo dos tempos. Atualmente, é a mais prevalente artrite inflamatória, especialmente em homens idosos (RICHETTE & BARDIN, 2010). Também considerada “a rainha das doenças” e a “doença dos reis”, foi um dos primeiros distúrbios a ser reconhecido como uma entidade clínica (WORTMANN, 2002) e está entre as condições agudas mais dolorosas que os seres humanos podem experimentar (MARTINON & GLIMCHER, 2006). Essa doença é geralmente associada com hiperuricemia e é caracterizada pelo depósito de cristais de urato de sódio (MSU) nas articulações (DALBETH & HASKARD, 2005). Embora exista uma variação significativa no desenvolvimento da gota, três estágios principais foram identificadas (KEITH & GILLILAND, 2007; MANDELL, 2008): o primeiro é caracterizado por uma hiperuricemia assintomática, ainda que muitas pessoas com aumento de ácido úrico no sangue não desenvolvem a doença (FEIG *et al.*, 2008); o segundo apresenta períodos de crises seguidos de períodos assintomáticos; e, finalmente, a gota intermitente aguda pode levar, ao longo dos anos, ao desencadeamento da gota tofácea crônica, onde há o aparecimento de nódulos duros geralmente depositados sob a pele ao redor das articulações (RICHETTE & BARDIN, 2010).

Nas últimas décadas, com o aumento da longevidade, da obesidade, da insuficiência renal crônica, do uso de diuréticos e aspirina e dos excessos alimentares ou alcoólicos, a prevalência da hiperuricemia e da gota aumentou significativamente (WORTMANN, 2005; BAKER *et al*., 2005). Porém, os medicamentos mais comumente aceitos, como a colchicina, os anti-inflamatórios não esteróides e o alopurinol têm limitações no seu uso, nomeadamente a intolerância, as alergias e as contra-indicações específicas de cada um (TERKELTAUB, 2003). Assim, o estudo dos mecanismos envolvidos na indução da inflamação que acompanha a gota é necessário para a busca por novos agentes terapêuticos mais efetivos e seguros para o tratamento da artrite gotosa.

A gota aguda é caracterizada por uma intensa reação inflamatória desencadeada por cristais de MSU, onde a interleucina 1-beta (IL-1β) desempenha um papel central nesse cenário, sendo que a sua produção se dá através da ativação do inflamassoma (MARTINON *et al*., 2006; MARTINON *et al*., 2010). Considerando esse mecanismo, drogas alvo para as vias regulatórias da ativação da IL-1β e do inflamassoma tornam-se importantes alternativas para o tratamento da

gota. De acordo com isso, tem sido mostrado que o tratamento com anti-IL-1 é efetivo para tratamento da gota (SO *et al*., 2007; TERKELTAUB *et al*., 2009), embora ainda apresente limitações em seu uso, como aumento da susceptibilidade a infecções, antigenicidade e custo elevado (BUSSO & SO, 2010).

Há pouco tempo, Lamkanfi e colaboradores (2009) mostraram que a glibenclamida, uma sulfoniluréia usada no tratamento da diabetes, inibiu a ativação do inflamassoma. Além disso, é atribuída à glibenclamida a modulação de alguns pontos importantes da resposta inflamatória, (SILVA-SANTOS *et al*., 2002; POMPERMAYER *et al*., 2007). Tais efeitos seriam decorrentes da capacidade da droga em bloquear canais de potássio sensíveis ao ATP. De fato, canais de potássio podem ser importantes na inflamação e seus inibidores como o tetraetilamônio podem ter efeitos antiinflamatórios. Porém, não se conhece o efeito da glibenclamida e de outros bloqueadores de canais de potássio na gota.

2. OBJETIVOS

2.1. Objetivo Geral

O objetivo do presente trabalho foi investigar o efeito antiinflamatório de um bloqueador de canal de potássio ATP-dependente (glibenclamida) em um modelo de ataque agudo de gota em ratos.

2.2. Objetivos Específicos

2.2.1. Verificar o efeito da glibenclamida na ação nociceptiva e edematogênica induzida por cristais de MSU em ratos;

2.2.2. Avaliar a ação da glibenclamida na infiltração de leucócitos induzida pelo MSU no líquido sinovial de ratos;

2.2.3. Verificar o efeito da glibenclamida na liberação de IL-1β induzida pelo MSU no líquido sinovial de ratos;

2.2.4. Verificar o efeito de diferentes concentrações de glibenclamida na liberação de IL-1β induzida pelo MSU em cultura de macrófagos;

2.2.5. Avaliar o efeito da glibenclamida na produção de prostaglandina E_2 induzida pelo MSU no líquido sinovial de ratos.

3. REVISÃO BIBLIOGRÁFICA

3.1. Artrite gotosa

3.1.1 Breve histórico

A gota está entre as mais antigas moléstias humanas conhecidas, tendo uma história de mais de 4.500 anos (TERKEUTALB, 2003). Inicialmente foi identificada pelos egípcios em 2640 antes de Cristo e chamada de podagra (palavra que significa dor no pé referindo-se a articulação metatarsofalangeal). Depois, foi reconhecida por Hipócrates no quinto século antes de Cristo, que se referiu a ela como “a doença incapacitante do andar.” Seis séculos após, Galeno descreveu os tofos em pacientes com gota, que são nódulos duros geralmente depositados sob a pele ao redor das articulações (NUKI & SIMKIN, 2006).

A história da gota foi continuamente associada ao consumo de comida e álcool em excesso. Devido a esse fato, essa doença foi relacionada a um estilo de vida que, pelo menos no passado, podia somente ser atribuído às pessoas abastadas, sendo denominada pela alcunha de “doença dos reis.” Essa característica era tão fortemente reconhecida, que a doença chegava a ser desejada, já que prevalecia entre a classe política e socialmente poderosa da época. A primeira epidemia de gota ocorreu durante o Império Romano, a segunda, durante o Império Britânico e talvez estejamos vivendo atualmente a terceira epidemia da civilização ocidental (FALASCA, 2006).

A aparência dos cristais de urato monossódico (MSU) foi descrita inicialmente em 1679, por Leeuwenhoek, que lhes descreveu como semelhantes a giz (partículas pequenas e transparentes com extremidades pontiagudas). A relação do ácido úrico à doença foi confirmada um século depois quando Wollaston (1797) demonstrou a presença de urato em um tofo de sua própria orelha. Por volta de 1859, Garrod declarou que o depósito do urato seria a causa e não a conseqüência da inflamação

ocasionada na gota. Faires e MacCarty estudaram a ligação dos cristais de urato monossódico com a gota, quando ambos, ao injetarem os cristais de MSU nas próprias articulações, desenvolveram uma rápida inflamação aguda que reproduziu todas as características de um ataque violento de gota. Embora este trabalho não tenha definido o mecanismo que iniciou o processo inflamatório, ele demonstrou que o cristal de MSU era o "disparador" inicial da artrite úrica (FAIRES & MC CARTY, 1962).

3.1.2 Epidemiologia

A gota é a mais prevalente artrite inflamatória nos países desenvolvidos, especialmente em homens idosos. A prevalência da doença é maior em homens e aumenta com a idade, sendo que nas mulheres, o desenvolvimento ocorre principalmente após a menopausa, justificado pela queda no nível de estrógenos (que são uricosúricos), aumentando a uricemia (HAK & CHOI, 2008). A prevalência aumenta com consumo excessivo de alimentos ricos em purinas, como a carne e os frutos do mar e a ingestão de bebidas alcoólicas, especialmente cerveja e destilados (CHOI *et al*., 2004a; CHOI *et al.,* 2004b). O consumo de derivados do leite, vitamina C e café estão associados com diminuição da uricemia ou prevalência da gota, ou ambos (CHOI *et al*., 2004; CHOI & CURHAN, 2007; GAO *et al*., 2008).

A incidencia de artrite gotosa vem aumentando nas últimas quatro décadas, principalmente em países industrializados. Muitos fatores parecem contribuir com esse evento como aumento da longevidade, da hipertensão, da obesidade, da síndrome metabólica, das doenças renais e do uso de alguns medicamentos, como diuréticos, ciclosporina e baixas doses de aspirina (WEAVER, 2008). O quadro ainda é intensificado devido às limitações encontradas no tratamento e estratégias

inadequadas para combater a inflamação e os altos níveis de ácido úrico (LAWRENCE *et al*., 1998). As estimativas revelam que no ocidente, mais de 1% dos homens adultos têm gota (MARTINON, 2010) e nos Estados Unidos, a prevalência de hiperuricemia e gota chega a 4% em pessoas acima dos 75 anos (WALLACE, 2004).

3.1.3 Fisiopatologia

O desencadeamento da gota está associado com o aparecimento de cristais de MSU no fluido sinovial, causando uma reação inflamatória (FIDDIS *et al.*, 1983).

O ácido úrico é um ácido fraco (pKa 5,8) que encontra-se em grande parte na sua forma ionizada (urato) em pH fisiológico. Este ácido é formado pela ação da enzima xantina oxidase sobre as purinas adenosina e guanosina (BANNASCH *et al*., 2008). Muitos mamíferos, mas não seres humanos, expressam a enzima uricase, que degrada o ácido úrico. O equilíbrio na quantidade de urato depende do balanço entre dieta, síntese e taxa de excreção. Quando tal equilíbrio não é mantido, os níveis de ácido úrico no sangue ultrapassam os valores de 7.0 mg/dl em homens e 6.0 mg/dl em mulheres, e com estes níveis considera-se instalada a hiperuricemia. Esse aumento na uricemia pode ser causado ou por superprodução ou por baixa excreção de ácido úrico. Na população em geral, 80 a 90% dos pacientes com gota são hipoexcretores (ROTT & AGUDELLO, 2003).

Alguns fatores, como temperatura corporal mais baixa (explicando ataques noturnos), mudanças no pH (por exemplo, cetose em pacientes durante o período pós-operatório) e nível de desidratação articular influenciam a solubilidade do urato no fluido articular (CHOI *et al.,* 2005). O urato depositado na articulação ou em tecidos peri-articulares tem a possibilidade de se ligar a um cátion sódio formando o

urato monosódico. O urato monosódico é mais solúvel do que o urato livre, porém seu limite de solubilidade é de 7 mg/dl a 37°C. Ocorre, então, a formação e precipitação de cristais de MSU nas articulações e em tecidos peri-articulares, pois em pacientes hiperuricêmicos a concentração deste sal se torna superior ao limite de solubilidade, embora se saiba que muitos pacientes hiperuricêmicos são assintomáticos e nunca desenvolverão gota (STRYER, 1996).

Os cristais de MSU são estímulos pró-inflamatórios que podem iniciar, amplificar e sustentar uma intensa reação inflamatória (CHOI *et al*, 2005; LIOTE & EA, 2006). Assim, os depósitos de cristais nas articulações são liberados e fagocitados por monócitos desencadeando uma típica resposta inflamatória, através da liberação de mediadores, assim como interleucina IL-1β, fator de necrose tumoral alfa (TNF-alfa) e interleucina 8 (IL-8) (PETRILLI & MARTINON, 2007). Os mecanismos pelos quais os cristais ativam as células nas articulações vêm sendo estudados (SO A., 2008; LIOTE & EA, 2007) e verificou-se que esta ativação pode ser desencadeada por 2 vias: uma via independente e outra via dependente de inflamossoma, mas a esta última tem sido atribuídas maiores implicações para a terapêutica. No citoplasma, o inflamassoma consegue detectar os cristais de MSU fagocitados por macrófagos, havendo a ativação da caspase-1, culminando com a maturação e secreção de IL-1β (MARTINON *et al*., 2006). A IL-1β secretada induz a síntese e/ou liberação de vários mediadores pró-inflamatórios, favorecendo o influxo de neutrófilos para a articulação. Resultados de estudos *in vivo* (CHEN *et al.,* 2006; MARTINON *et al*., 2006; PETRILLI & MARTINON, 2007) tem confirmado que IL-1β e esta via estão associadas com a resposta inflamatória induzida por MSU, sugerindo que IL-1β é o mediador principal da inflamação em gota aguda e um alvo chave para a terapêutica.

Os cristais de MSU podem estimular células por outras maneiras, independente da via do inflamassoma; através do contato direto (cristal-célula), podem promover ativação celular e desencadear a liberação de vários mediadores inflamatórios, assim como as prostaglandinas, que desempenham importante papel no desenvolvimento da resposta inflamatória vista na gota (AKAHOSHI *et al*., 2007).

3.1.4. Características clínicas

Embora exista uma variação significativa de paciente para paciente no desenvolvimento de gota, três fases principais foram identificadas (MANDELL, 2008; KEITH & GILLILAND, 2007). A primeira fase é a hiperuricemia assintomática, uma anormalidade fisiológica comum que está fortemente associada com a gota. Surpreendentemente, a maioria das pessoas com hiperuricemia não desenvolvem gota, e este aumento de ácido úrico no sangue tem sido associado com hipertensão, diabetes, doença renal e doença cardiovascular. O papel do ácido úrico ou cristais de ácido úrico nestas doenças ainda não está claro (FEIG *et al*., 2008).

A segunda etapa é caracterizada por ataques periódicos seguidos por períodos assintomáticos. Esses pacientes geralmente desenvolvem febre, calafrios e dor que se estende ao longo de um período de 6 a 12 h. Depósitos de cristal e liberação nas articulações causam inchaço e eritema. Nesta fase, a apresentação clínica inicial mais comum é a monoartrite aguda. A articulação mais frequentemente atingida é a primeira metatarso-falângica (50% dos casos), designando-se a crise de podagra. No entanto, podem ser envolvidas outras articulações, como as articulações do tarso, tibiotársicas, joelhos, punhos ou qualquer metacarpo-falângica. A crise começa geralmente de madrugada exibindo os sintomas clássicos da inflamação. Os sintomas de edema, calor, rubor e dor continuam durante vários

dias, marcando o influxo de neutrófilos juntamente com monócitos e outros leucócitos pró-inflamatórios para a área. Mesmo na ausência de tratamento, os sintomas inflamatórios desaparecem dentro de 7 a 10 dias (RICHETTE & BARDIN, 2010), sendo que em torno de 60% dos indivíduos que tiveram o primeiro ataque de gota irão ter uma segunda experiência no intervalo de 1 ano (FERRAZ & O'BRIEN, 1995).

Finalmente, a gota intermitente aguda pode levar, ao longo dos anos, à gota tofácea crônica (RICHETTE & BARDIN, 2010). Os ataques seguem por diversos anos, aonde as crises vão se tornando cada vez mais frequentes e intensas. Esse aumento na intensidade pode ser acompanhado pela formação de tofos (aglomerados de cristais MSU e células imunes nas articulações ou tecidos moles). A formação de tofos ao redor das articulações leva a erosão óssea e deformações, podendo evoluir para uma forma debilitante (NAKAYAMA *et al*., 1984).

3.1.5. Tratamento

O tratamento da gota apresenta três enfoques principais: o primeiro está baseado em um estilo de vida mais apropriado, objetivando o controle do ácido úrico no sangue através de ingestão moderada; o segundo envolve as drogas que são capazes de diminuir a uricemia e o terceiro são anti-inflamatórios utilizados para amenizar os sintomas da inflamação associados ao ataque de gota (CHOI *et al*., 2004a; CHOI *et al*., 2004b). O uso de anti-inflamatórios inclui os anti-inflamatórios não esteroidais (AINES) e os glicocorticoides (RIDER & JORDAN *et al*., 2009). Os AINES podem ser utilizados tanto como profiláticos ou para aliviar os sintomas da inflamação, assim como a colchicina, um componente ativo da planta *Colchicum autumnale* (ROBERGE *et al.,* 1993). Assim, os AINES apresentam uma limitação no

uso, devido aos possíveis efeitos colaterais, como perda da função renal, retenção de líquido, gastro e hepatopatia e função cognitiva prejudicada (GONZALEZ *et al*., 1994) e a colchicina, mesmo sendo a mais antiga opção de tratamento para gota, pode apresentar efeitos gastrointestinais adversos e toxicidade que aumenta em pacientes idosos, com doença hepática ou renal (AGUDELO & WISE 1998). Finalmente, os glicocorticóides são eficazes no tratamento da gota, sendo particularmente apropriados como estratégia de tratamento em pacientes com doença renal crônica (TERKELTAUB, 2003); se usados de uma forma adequada, em um curto prazo, são considerados uma alternativa segura, principalmente quando os AINES forem contraindicados. Nos ataques monoarticulares, uma injeção intra-articular com um corticosteroide de longa duração é, muitas vezes, a maneira mais segura de tratamento. Ainda vale lembrar que os corticosteroides são contraindicados em pacientes diabéticos, já que favorecem a gliconeogênese hepática (ROTT & AGUDELO, 2009).

Os níveis de ácido úrico podem ser controlados por drogas que baixam a sua produção (drogas uricostáticas) ou que aumentem a sua excreção renal (drogas uricosúricas). Alopurinol é o agente uricostático mais comumente prescrito e age inibindo a enzima xantina oxidase, com efeitos indesejáveis pouco frequentes, embora possam ser severos, ocorrendo frequentemente em pacientes com insuficiência renal (TERKELTAUB, 2003; ANZAI *et al.,* 2008). Outra limitação ao uso do alopurinol é o desenvolvimento de hipersensibilidade em torno de 2% dos pacientes (BECKER *et al.,* 2005) e em cerca de 20% ocorre intolerância ou refratariedade para a droga (ZHANG *et al.,* 2006). Em 2009, outro inibidor da xantina oxidase foi aprovado pelo FDA, febuxostate, apresentando efeitos colaterais de menor intensidade, quando comparado ao alopurinol (NEOGI, 2011).

As drogas uricosúricas tem a função de prevenir a reabsorção do ácido úrico nos rins, favorecendo sua excreção. Exemplos dessas drogas são probenecida e benzbromarone (ENOMOTO *et al*., 2002). Esta classe de medicamentos é menos usada que os inibidores da xantina oxidase e é contraindicada na presença de nefrolitíase (REINDERS *et al.,* 2009).

Devido principalmente aos importantes efeitos indesejados e aos pacientes não respondedores às drogas tradicionais, novas tentativas terapêuticas têm surgido para amenizar os sintomas da gota. Pegloticase, uma uricase peguilada, foi aprovada em 2010 pelo FDA para o tratamento de gota crônica, entretanto as reações na administração foram comuns, uma vez que a via recomendada é a intravenosa (SUNDY *et al.,* 2008). A imunogenicidade das uricases também tem sido um fator limitante para o uso, já que é frequente o desenvolvimento de anticorpos contra estes fármacos, mesmo usando a peguilação da enzima (TERKELTAUB, 2010).

Outra alternativa tem sido usada com mais êxito, os agentes anti-citocinas, especialmente anti-IL-1 (SO *et al*., 2007; TERKELTAUB *et al*., 2009; NEOGI 2010; SO *et al*., 2010). Esta nova terapia está baseada na importância da IL-1 na patogênese da gota; desta maneira, acredita-se que a sua inibição possa extinguir os ataques de gota aguda. Assim, drogas anti-IL-1 (anakinra, canakinumad e rilonacept) têm sido usadas com sucesso tanto no tratamento quanto na profilaxia da doença (SO *et al*., 2007; SO *et al.,* 2010).

3.2 Inflamassoma, IL-1β e gota

O termo inflamassoma descreve um complexo proteico citoplasmático de elevado peso molecular composto de uma proteína da família NLRP (ou NALP), uma

proteína adaptadora ASC e uma caspase inflamatória (BUSSO & SO, 2010) e exibe uma atividade enzimática que ativa a IL-1β (MARTINON *et al*., 2002). Tal atividade depende do recrutamento de caspases inflamatórias ao complexo ativo (MARTINON & TSCHOPP, 2004; NADIRI *et al*., 2006). A caspase inflamatória melhor caracterizada é a caspase-1, que tem como principais substratos as citocinas, tais como IL-1β e IL-18, dois conhecidos mediadores da resposta inflamatória que necessitam de processamento para se tornarem ativas (MARTINON, 2010). A IL-1β, também conhecida como pirógenio endógeno, é uma citocina inflamatória cuja produção é controlada por pelo menos três passos distintos (BURNS *et al*., 2003). O primeiro passo envolve a produção da proteína pro-IL-1β, a qual é seguida por clivagem para produzir a proteína IL-1β ativa, e, finalmente, ocorre o terceiro passo, quando acontece a liberação para o meio extracelular. O passo intermediário, o processamento da pro-IL-1β, envolve a ativação do inflamassoma (MARTINON *et al.,* 2002; MARIATHASAN *et al*., 2004). Desde a sua descoberta, a desregulação do inflamassoma tem sido associada a numerosas doenças, causadas por mutações em seus reguladores, ou por sua ativação exagerada, como na gota, onde é mediada pela deposição local de cristais (MARTINON *et al*., 2006).

Ao nível molecular, a inflamação gotosa pode ser dividida em duas fases (MARTINON, 2006). A primeira fase (de ativação do inflamassoma) requer células fagocíticas, tais como monócitos ou macrófagos capazes de “perceber” os cristais e ativar o inflamassoma NLRP3. Esta fase aciona o processamento e liberação de IL-1β ativa. Liberação de IL-1β, então, ativa a segunda fase, envolvendo as células que expressam o receptor para IL-1. A ligação da IL-1β a suas células-alvo (possivelmente sinoviócitos) ativa fatores de transcrição tais como NF-kB. Este estímulo transcricional promove a produção e liberação de mediadores inflamatórios,

assim como quimiocinas que agem no recrutamento de neutrófilos (MARTINON & GLIMCHER, 2006 (Figura 1). Desta maneira, tem sido colocado em evidência o papel crucial da IL-1β na inflamação gotosa (CHEN *et al*., 2006), sendo que pesquisadores têm apontado a terapia anti-IL-1 como uma alternativa ao tratamento antiinflamatório convencional para gota (SO *et al*., 2007; TERKELTAUB *et al*., 2009; SO *et al*., 2009; SCHUMACHER *et al*., 2009; SO *et al*., 2010; TERKELTAUB *et al.,* 2010).

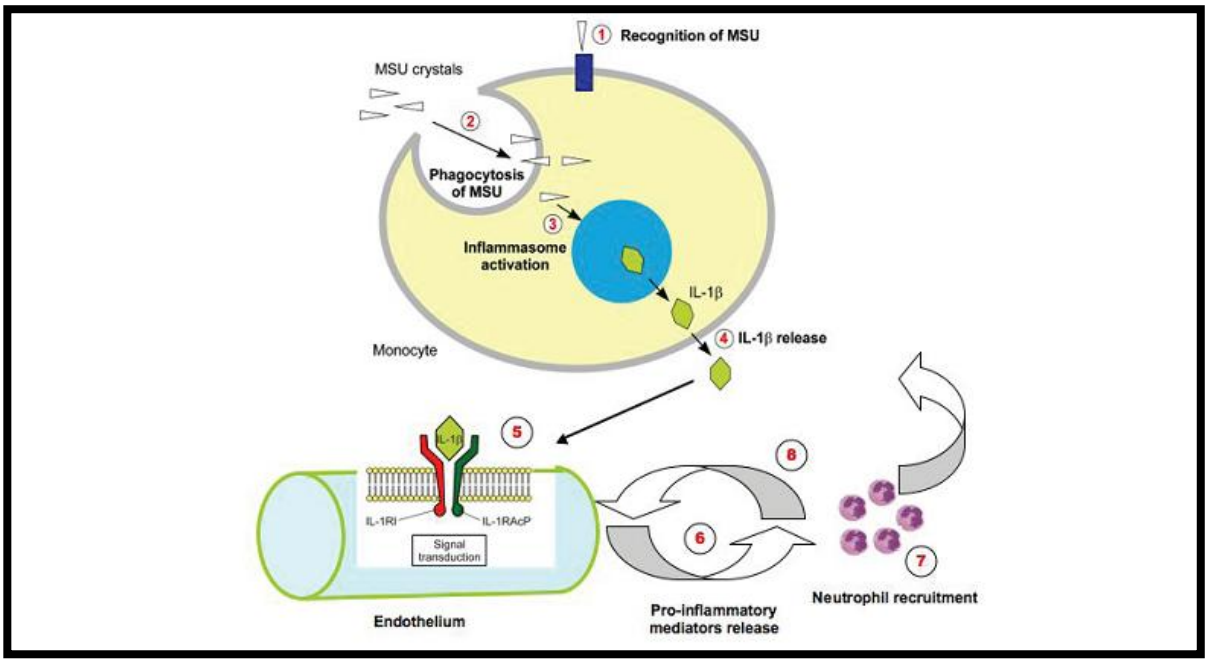

Figura 1: Fases da ativação do inflamassoma na artrite gotosa (Fonte: Busso e So, 2010).

Embora trazendo expectativas interessantes, a inibição da IL-1β pode apresentar efeitos indesejáveis, pois os efeitos bloqueadores dos anti-IL-1 são amplos, inibindo todas as ações da interleucina em todos os tecidos, não somente no sítio de inflamação. Desta forma, além da ação antiinflamatória local essa intervenção terapêutica poderia causar uma imunossupressão sistêmica, aumentando a suceptibilidade dos pacientes à infecção. Além disso, os anti-IL-1 tem outras limitações como antigenicidade e custo (BUSSO & SO, 2010). Assim

fármacos que conseguissem bloquear as ações da IL-1 somente no sítio da inflamação e que fossem seguros e de baixo custo seriam muito interessantes para o tratamento da gota.

Glibenclamida, uma sulfoniluréia amplamente utilizada no tratamento da diabetes, foi testada, recentemente, para avaliar seu efeito sobre o inflamassoma. Assim, Lamkanfi e colaboradores (2009) mostraram que a glibenclamida foi capaz de inibir a ativação do inflamassoma NALP3 e a maturação de IL-1β e IL-18 através da caspase-1. Isso é bastante interessante, já que estudos têm revelado que o inflamassoma desempenha um papel chave na intensa reação inflamatória característica nos ataques de gota (PUNZI *et al*., 2012). Assim, fármacos que visam inibir a atividade do inflamassoma podem, portanto, ser de interesse e oferecer novas possibilidades para o tratamento eficaz e específico de inflamassomopatias, assim como a gota. Desta forma, a glibenclamida, por ser uma droga já utilizada na clínica, bem tolerada, com poucos efeitos colaterais e com reduzido custo, poderia ser útil para pacientes com artrite gotosa, porém seu efeito em modelos de gota ainda não foi testado. Além disso, já foi mostrado que a glibenclamida desempenha um efeito antiinflamatório em alguns modelos (SILVA-SANTOS *et al*., 2002; POMPERMAYER *et al.,* 2007), e essa ação seria dependente do bloqueio seletivo a canal de potássio sensível a ATP (K_{ATP}), exercido por esta droga.

3.3 Canais de potássio e inflamação

O potencial de membrana é determinado pela sua permeabilidade diferencial aos diversos íons e pela distribuição relativa destas espécies iônicas (sódio, cloreto, cálcio, potássio, etc) em cada um dos compartimentos que a membrana separa. Dentre os canais iônicos, os canais de potássio (KC) representam um diversificado

grupo encontrado na maioria das células eucarióticas de animais e plantas e permitem o movimento dos íons K^+ através da membrana plasmática (JAN & JAN, 1992). Normalmente, existe uma alta concentração de K^+ no meio intracelular (~140 mEq/L) e uma baixa concentração no meio extracelular (~4 mEq/L), havendo uma permeabilidade de repouso, porque os KC estão abertos. Os canais são importantes para regulação da função celular de células excitáveis e não excitáveis. Através da sua abertura, eles estabilizam o potencial de membrana, e em células excitáveis tal abertura irá restabelecer o potencial de repouso, repolarizando e, portanto finalizando o período do potencial de ação, disparado anteriormente. Em células não excitáveis, os canais de K^+ também apresentam função importante na regulação do volume celular, transdução de sinal e manutenção do potencial de repouso (GRISSMER *et al.,* 1997).

Diferentes tipos de KC foram identificados, sendo divididos em três classes principais: os KC controlados por voltagem, os KC com domínio de dois poros e os KC retificadores de entrada, sendo que os canais de K_{ATP} pertencem a esta última família (RANG & DALE, 2007). Os canais K_{ATP} foram assim chamados devido à sensibilidade ao ATP intracelular, que os fecha. Durante o estresse metabólico, os níveis de ATP caem e os de ADP aumentam, promovendo abertura substancial desses canais, nessas condições. Além do metabolismo celular, esses canais são regulados por outros fatores como quinases, fosfatases e proteínas G (para revisão, ver BUCKLEY *et al*., 2006).

Os canais K_{ATP} são um complexo de pelo menos duas proteínas, o receptor de sulfoniluréias (SUR) associado a uma subunidade formadora do poro que pertence à família Kir. A função melhor caracterizada destes canais é o controle da liberação de insulina pelas células β pancreáticas. Durante o jejum, os canais são

mantidos abertos devido à baixa glicemia. Os níveis de glicose aumentam após o desjejum, elevando o ATP intracelular, resultando em fechamento dos canais K_{ATP} e despolarização da membrana das células β. Isso por sua vez, abre canais de cálcio dependentes de voltagem fazendo com que o cálcio entre na célula, o que leva à exocitose de grânulos que contem insulina, estimulando, assim, a sua liberação (SEINO & MIKI, 2003). De uma maneira similar ao ATP, as sulfoniluréias bloqueiam o canal, estimulando a secreção de insulina por se ligarem a subunidades SUR, desempenhando, assim, um efeito hipoglicemiante (STANDEN, 1997).

Adicionalmente, é atribuída à glibenclamida, uma sulfoniluréria de segunda geração, a influência em alguns pontos importantes da resposta inflamatória, assim como a supressão da migração de neutrófilos e quimiotaxia durante uma reação inflamatória aguda induzida por carragenina (SILVA-SANTOS *et al*, 2002) e a inibição do aumento da permeabilidade vascular associado com reperfusão pós-isquêmica (POMPERMAYER *et al.*, 2007). Tais ações seriam decorrentes do efeito inibitório da droga sobre os canais de K_{ATP}. Porém, não se sabe se a inibição do inflamassoma poderia participar destes efeitos.

Além dos canais de K_{ATP}, parece que os demais canais de potássio também desempenham um importante papel na sinalização intracelular de cascatas inflamatórias, sendo que tanto a expressão como a funcionalidade de alguns subtipos de canais de K^+ pode ser modificada após um estímulo inflamatório (AUTIERI *et al*., 1997; CZAIKA *et al*., 2000). Além do mais, já foi demonstrado que altas concentrações de K^+ previnem a liberação de IL-1β, sugerindo que a redução da concentração intracelular de K^+ é necessária para o processamento da IL-1β, através da ativação da enzima caspase-1 (PERREGAUX *et al.,* 1994; WALEV *et al.,* 1995; CHENEVAL *et al*., 1998; KAHLENBERG & DUBYAK, 2004). Esses resultados

se somam a outros que afirmam que o bloqueio dos KC pode inibir a ativação de macrófagos (MARUYAMA *et al*., 1994; WALEV *et al*., 1995) implicando na redução da produção de citocinas (BLUNCK *et al*., 2001; PAPAVLASSOPOULOS *et al.,* 2006). Em adição, sabe-se que o óxido nítrico (NO) é capaz de modular a atividade dos KC, assim como esses canais parecem ter importante papel na sinalização da expressão da NO sintase-2 (NOS-2) e consequente produção de NO (LOWRY *et al*., 1998; WU *et al*., 1998). De acordo com isso, o tetraetilamônio (TEA), um bloqueador não seletivo de KC (SORDI *et al.*, 2010), tem indicado efeitos benéficos em modelos de sepse, assim como a redução de parâmetros inflamatórios, visto através da diminuição da atividade da enzima mieloperoxidase e da expressão da enzima NOS-2, assim como da redução dos níveis de nitrito+nitrato, de TNF-α e de lL-1β (SORDI *et al.*, 2011). Em contrapartida, em um modelo de edema de pata em camundongos, o TEA reduziu o efeito antiedematogênico desempenhado pelo NO (FERNANDES & ASSREUY, 2004).

Dessa forma, observamos que os dados da literatura são vastos e ilustram o possível envolvimento de diferentes tipos de KC nas alterações vistas nas reações inflamatórias. Estes dados permitem especular que estas alterações também podem acontecer durante o desencadeamento da artrite gotosa, e estes efeitos podem inclusive ser modulados por bloqueadores ou abridores destes canais.

4. RESULTADOS

4.1. Manuscrito: Efeitos antinociceptivo e anti-edematogênico da glibenclamida em um modelo de gota aguda em ratos.

Título:

Anti-nociceptive and anti-edematogenic effects of glibenclamide in a model of acute gouty attack in rats

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Anti-nociceptive and anti-edematogenic effects of glibenclamide in a model of acute gouty attack in rats

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Running title: Glibenclamide in gout pain and edema

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ABSTRACT

Objective and design: We investigated the effect of glibenclamide on inflammatory parameters in a model of acute gouty attack in rats.

Treatment: Intra-articular injection of 50 µl of monosodium urate (MSU) crystals (1.25 mg/site) was used to induce gout-related inflammation. The effects of glibenclamide (1-10 mg/kg, s.c.) or dexamethasone (8 mg/kg, s.c., positive control) were assessed on several inflammation parameters.

Methods: Spontaneous nociception assessment, edema measurement, total and differential leucocyte counts, interleukin (IL)-1β release, prostaglandins (PGE_2) production and determination of blood glucose levels were analyzed. In macrophages culture was measured the IL-1β levels. Statistical significance was assessed by one- or two-way analysis of variance.

Results: Glibenclamide (3 mg/kg) or dexamethasone (8 mg/kg) prevented nociception and edema induced by MSU injection in rats. The treatment with glibenclamide (3 mg/kg) did not affect leukocyte infiltration, IL-1β release and PGE_2 production, however, glibenclamide (200 µM) reduced the production of IL-1β by MSU-stimulated macrophages. Dexamethasone significantly reduced leukocyte infiltration, IL-1β release and PGE_2 production. Glibenclamide (3 mg/kg) reduced and dexamethasone increased blood glucose levels of MSU-injected rats.

Conclusions: Glibenclamide reduced nociception and edema, but not leukocyte infiltration, IL-1β release and PGE_2 production. Its substantial effect on nociception and edema suggests that glibenclamide can be an interesting option as an adjuvant treatment for pain induced by acute attacks of gout.

Key words: nociception; edema; leukocyte; inflammasome; ATP-sensitive potassium channel; interleukin-1β.

1. INTRODUCTION

Gout is a metabolic disorder associated with an excess of circulating uric acid resulting in the deposition of monosodium urate (MSU) crystals in tissues. After formed, MSU crystals may be deposited in joints, usually in the big toe or ankle, causing neutrophil infiltration, swelling and excruciating pain [1, 2]. Estimations from the Third National Health and Nutrition Examination Survey (NHANES III) indicate that 0.5% of total population suffered from a gout attack [3]. In addition, gout is currently considered the most common inflammatory arthritis in men over 40 years old, exceeding rheumatoid arthritis [4, 3]. Although gout is a potentially treatable disease, its diagnosis and treatment are poorly managed worldwide [5, 6].

In the last decade, the complex NLRP3 inflammasome has been associated with the regulation of the innate inflammatory phenotype of several diseases, including gout [7]. The NLRP3 inflammasome is a molecular platform activated by pathogens as well as danger signals, including MSU, which triggers activation of inflammatory caspases, such as caspase-1, to generate the biologically active IL-1β from pro-IL-1β [8, 9, 10]. IL-1β level is elevated in synovial fluid and thus contributes to local inflammation in gout patients [11]. Considering this mechanism, drugs targeting NLRP3 inflammasome may have a favorable effect on gout, which may provide a new perspective for its treatment [12].

Glibenclamide (glyburide) is the most widely used sulfonylurea for the treatment of type 2 diabetes [13]. The relevant pharmacological drug action in diabetes is the inhibition of K_{ATP} channels in pancreatic β cells leading to stimulation of insulin secretion [14]. Recently, a study demonstrated that glibenclamide inhibits NLRP3 inflammasome activation caused by a variety of stimuli in macrophages *in*

vitro [15]. However, the ability of glibenclamide to inhibit NLRP3 inflammasome *in vivo* is currently unknown.

Since NLRP3 inflammasome activation seems to be important for gout development and considering that glibenclamide may inhibit its activation, the aim of the present study was assess the anti-nociceptive and anti-inflammatory effects of glibenclamide in an *in vivo* model of acute gout attack induced by intra-articular MSU injection in rats.

2. MATERIALS AND METHODS

2.1. Animals

The experiments were performed using adult male Wistar rats weighing 250–300 g (in vivo) and 3-4 month old C57Bl6 mice (in vitro experiments). Animals were housed under controlled temperature (22 ± 1 °C), on a 12 hour light/12 hour dark cycle and with standard lab chow and water *ad libitum*. Rats were acclimated to the experimental room for at least 1 hour before the experiments. The present study was conducted in accordance with the internationally accepted principles for laboratory animal use and care. Our Institutional Ethics Committee approved all procedures (process number UFSM 23081.003640/2009-61). The number of animals and nociceptive stimuli were the minimum necessary to demonstrate the consistent effects of drugs treatments.

2.2. Drugs and reagents

Synthetic monosodium urate (MSU) crystals were prepared as described previously [16]. Briefly, 4 g of uric acid (Vetec®, Brazil) was dissolved and heated in 800 mL of H_2O, adjusted to pH 8.9 with NaOH (9 mL, 0.5 N) at 60 °C, cooled

overnight in a cold room. Needle-like crystals were recovered and suspended in phosphate-buffered saline (PBS; 10.71 mM K_2HPO_4, 6.78 mM NaH_2PO_4, 120.4 mM NaCl; pH 7.4). Polarized light microscopic examination confirmed that the crystals were rod-shaped and varied in length (12 ± 2 µm). The preparation was endotoxin free as determined by an amebocyte cell lysate assay (Sigma®, St Louis, USA). Glibenclamide was purchased from Sigma® (St Louis, USA), and the stock solution was prepared in 95% DMSO and 5% Tween 80. Dexamethasone was purchased from Aché® (São Paulo, Brazil). Thioglycollate medium, Dulbecco modified Eagle's medium (DMEM), calf serum and antibiotics were purchased from Gibco (São Paulo, SP, Brazil).

2.3. MSU crystals-induced arthritic gout and treatments

The experiments were conducted in accordance with the method described by Coderre and Wall [17], with some modifications. Briefly, 50 µL of vehicle (PBS) or MSU suspension (1.25 mg/site) were injected intra-articularly (i.a.) into the tibio-tarsal joint (ankle) on isoflurane-anesthetized animals. The animals were treated subcutaneously (s.c.) with vehicle (90% PBS plus 9.5% DMSO and 0.5% Tween; 1 ml/kg), dexamethasone (8 mg/kg, used as positive control) or glibenclamide (1-10 mg/kg) immediately after crystal injection. The dosage and time of treatment were based on our previous studies [18, 19].

2.4. Nociception assessment

To assess nociception caused by intra-articular of MSU, rats were habituated to observation in glass boxes and the spontaneous nociception was assessed using a standing paw pressure score from 0 until 3, as previously described by Coderre and Wall [17]. The amount of weight that the rat was willing to put on the hindpaw of the injected limb was evaluated and categorized according to the scale described

below: score 0 was considered when the paw pressure was normal, with equal weight on both hind-paws; score 1 was considered when the paw pressure was slightly reduced, with the paw completely on the floor, but toes are not spread; 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 foot elevated completely.

2.5. Edema measurement

The edema formation was assessed as an increase in ankle thickness, in millimeter (mm) after MSU injection and measured with a digital caliper [16], compared to baseline values. Results were expressed as variation of edema (difference between the measurement of ankle thickness before and after treatment).

2.6. Collection of synovial fluid, leucocyte count, IL-1β and prostaglandin (PGE_2) determinations

Six hours after MSU injection, animals were anaesthetized with sodium pentobarbital (100 mg/kg, i.p.) and perfused with saline to avoid blood interference. Injected ankle synovial cavities were washed with 60 µL of PBS (three times of 20 µL each) by inserting a tip into the rat ankle joint, the synovial washes being recovered by aspiration [20]. To determine whether the treatment with glibenclamide induced changes in leukocyte numbers, IL-1β and PGE_2 levels, synovial fluid samples from animals treated with vehicle, glibenclamide (3 mg/kg) or dexamethasone (8 mg/kg) were subjected to total and differential leukocyte counts and IL-1β and PGE_2 determinations. Concentrations of the IL-1β were quantified by an enzyme-linked immunosorbent assay (ELISA) kit, according to the manufacturer's instructions (PeproTech®, Rocky Hill, USA). PGE_2 assay was carried out using ELISA assay

according to the manufacturers' instructions (Cayman Chemical Company®, Ann Arbor, USA). Total leucocyte counts were performed in a Neubauer chamber after dilution of the synovial fluid samples in Turk's solution (0.01% Gentian violet and 1% glacial acetic acid in distilled water) under an optical microscope. Differential neutrophil counts were performed using May–Grunwald–Giemsa-stained cytospins (Cytospin 248, FANEM®, São Paulo, Brazil) and values are expressed as numbers of cells per cavity ($\times 10^6$).

2.7. Macrophage isolation and IL-1β assay

Mice C57Bl6 were injected i.p. with 2 ml of sterile thioglycollate medium (Becton Dickinson, Franklin Lakes, NJ, USA). Four days later, peritoneal macrophages were collected by lavage with cold Dulbecco's modified Eagle's medium (DMEM) and were centrifuged (1200 g; 10 min). Then, the cells were suspended in DMEM and 10% fetal bovine serum, seeded in 96-well plates (1×10^6 cells/ml), incubated for 90 min in a humidified atmosphere of 5% CO2 at 37 °C and non-adherent cells were removed by washing. Twenty-four hours after, macrophages were incubated for 30 min with glibenclamide (1, 50 and 200 μM), and then, MSU (300 μg/ml) was added without washing. Twenty-four hours later, the supernatant were obtained and assayed for IL-1β using commercially available ELISA kits according to the manufacturer's recommendations (PeproTech Inc®, Rocky Hill, USA).

2.8. Determination of blood glucose levels

Blood samples of animals treated with vehicle, glibenclamide (3 mg/kg) or dexamethasone (8 mg/kg) were collected from the tail vein 6 h after injection. Glucose levels were assessed by Accu-Chek Active W monitoring kit (Roche Diagnostics®, Indianapolis, USA).

2.8. Statistical analysis

All values are expressed as mean ± S.E.M. The statistical significance between groups was assessed by one- or two-way analysis of variance (ANOVA), when appropriate. Pos-hoc tests (Dunnett's test for one-way or Bonferroni for two-way ANOVA) were also carried out when appropriate. P values lower than 0.05 ($P < 0.05$), 0.01 ($P < 0.01$) or 0.001 ($P < 0.001$) indicated statistically significant differences.

3. RESULTS

3.1. Effects of glibenclamide on nociception and edema induced by MSU

MSU produced nociception and edema when injected into the rat ankle joint (Fig. 1A and B). Treatment with glibenclamide (3 mg/kg, s.c.) or dexamethasone (8 mg/kg, s.c., used as a positive control) reduced the spontaneous nociception and the edema induced by MSU.

The anti-nociceptive effect of glibenclamide started 4 h after injection, peaked and lasted up to 6 h after administration (Fig. 1A). The anti-nociception produced by dexamethasone was observed as early as 2 h, peaked and lasted up to 6 h after injection (Fig. 1A). Therefore, glibenclamide presented an anti-nociceptive effect with an efficacy similar to that of dexamethasone, the maximal inhibitory effect being of 67 ± 11% and 70 ± 7% for glibenclamide and dexamethasone, respectively.

The anti-edematogenic effect of glibenclamide or dexamethasone was long-lasting, beginning at 6 and 4 h, respectively, and still present up to 24 h after treatment (Fig. 1B). Dexamethasone displayed a higher efficacy compared to glibenclamide in reducing MSU-induced edema, with an inhibitory effect of 77 ± 7% and 28 ± 7%, respectively.

When dose-response curves were carried out at the peak effect (6 h), glibenclamide reduced MSU-induced edema with a similar efficacy with the doses of 1 and 3 mg/kg (31 ± 7% and of 28 ± 7% inhibition). However, glibenclamide decreased the nociception only with the dose of 3 mg/kg (inhibition 67 ± 11%; Fig. 2A and B). Furthermore, glibenclamide did not cause any alteration in spontaneous nociception or ankle thickness when subcutaneously administered in animals that received intra-articular injection of PBS (data no shown).

Since only the dose of 3 mg/kg of glibenclamide given 6 h after MSU injection was able to prevent both nociception and edema, this dosage and time of observation were chosen for the next set of experiments.

3.2. Effects of glibenclamide on leukocyte infiltration, IL-1β release and PGE_2 production induced by MSU

In order to investigate the inflammatory response induced by MSU crystals and if the treatment with glibenclamide (3 mg/kg) or dexamethasone (8 mg/kg) would alter the leukocyte infiltration and IL-1β and PGE_2 production, the synovial fluid of the ankle joint was collected and the accumulation of inflammatory cells and the concentrations of IL-1β and PGE_2 were determined. The number of infiltrating cells was substantially increased by about 3-fold 6 h after MSU crystal injection (Fig. 3A). Additionally, MSU crystals induced migration of leukocytes, mostly polymorphonuclear cells (Fig. 3B). Neither the increase in leukocyte number nor the profile of migrated cells was changed by glibenclamide (Fig. 3A and B). On the other hand, dexamethasone significantly reduced total leukocyte infiltration (92 ± 4% reduction).

MSU crystals also increased IL-1β and PGE_2 concentrations in the synovial

fluid (Fig. 4 and Fig. 5, respectively). Glibenclamide treatment did not affect these increases whereas dexamethasone fully inhibited MSU-induced IL-1β and PGE_2 release (Fig. 4 and Fig. 5, respectively).

3.3. Effects of glibenclamide on IL-1β release in cultured macrophages

IL-1β was assayed in the supernatants of cultured peritoneal macrophages obtained from C57-Bl6 mice, incubated in the presence or absence of MSU. As depicted in Fig. 6, MSU increased levels in the supernatant of peritoneal macrophages. Treatment with glibenclamide (1 and 50 μM) did not affect this increase. Glibenclamide was able to affect IL-1β only when at very high concentration (200 μM).

3.4. Effects of glibenclamide on blood glucose levels on MSU injected animals

MSU injection in the ankle joint did not alter the glucose levels, when compared to animals that received PBS (Fig. 7). On the other hand, the administration of glibenclamide reduced blood glucose levels of rats by 23 ± 2% when compared to MSU/vehicle group but dexamethasone increased blood glucose levels of MSU- injected animals.

4. DISCUSSION

Recent large epidemiologic studies from different countries have reported gout prevalence of about 3.8 % in the adult population, with increasing prevalence in the past two decades [21, 22]. Moreover, patients with acute and chronic gouty arthritis have lower health-related quality of life due to significant pain, limitation of activity, and disability [23]. Despite these problems, treatment options for gout have remained essentially the same for the last 50 years making mandatory the discovery of new

targets and treatments for this disease [7]. Our study demonstrates that glibenclamide, a drug already in the clinical practice, reduced nociception and edema in a model for acute gout attacks, namely the injection of MSU crystals in rats. Interestingly, glibenclamide did not affect leukocyte infiltration or IL-1β release induced by MSU.

During an acute gouty attack episode, the affected joint exhibits all of the classic symptoms of inflammation including intense pain in the affected joint [2]. The severity of the pain can be so excruciating that some patients expressed the desire to amputate the affected limb to alleviate it [24]. We observed that rats submitted to intra-articular injection of MSU, but not PBS, presented nociceptive behavior (with a peak from 4 to 6 h after MSU administration). These findings are in accordance with the clinical findings, where acute gouty arthritis is associated with severe pain escalating over a to 12 h period [25], with the peak of spontaneous pain after MSU injection in human subjects occurring at 4 h [26].

When we analyzed the anti-nociceptive effect of glibenclamide in rats submitted to intra-articular injections of MSU, it was observed that glibenclamide presented anti-nociceptive effects with an efficacy similar to dexamethasone. Distinctly from our findings, literature data using other models of pain and nociception demonstrated that glibenclamide has no anti-nociceptive effect per se, but reverses the effect of analgesic drugs by a mechanism dependent on K_{ATP} channels in sensory neurons [27, 28, 29, 30]. Therefore, the action of glibenclamide in the nociception induced by MSU could be mediated by a mechanism different from K_{ATP} channel blockade in sensory neurons, including inflammasome inhibition.

Another important clinical sign of acute gout attacks is the articular edema, caused by the plasma extravasation [31]. In this study, glibenclamide and

dexamethasone presented anti-edematogenic effect, being dexamethasone more effective than glibenclamide in reducing MSU-induced edema. Our results are in accordance with previous studies showing that glibenclamide inhibited the increase in vascular permeability associated with reperfusion injury [32] or induced by carrageenan in rats [18].

Clinically, the inflammatory symptoms in the gout are maintained by a substantial influx of neutrophils – a key cell in gout – along with monocytes and other pro-inflammatory leukocytes into the area [10]. In accordance with literature, we observed that MSU produced an extensive leukocyte infiltration in the synovial fluid of rats, composed mostly by neutrophils [31]. Some studies have shown that glibenclamide suppressed neutrophil migration and chemotaxis during acute inflammatory responses by a mechanism possibly dependent on its K_{ATP} channel blocking activity [18, 32]. Moreover, it was suggested that neutrophils are the major cellular targets for the action of the drug [32]. Our results show that glibenclamide did not affect the increase or the composition of the leukocyte migration. One possibility to explain the differential effects of glibenclamide would be that our model affects primarily a joint and not an open cavity or an organ. These differences highlight that distinct mechanisms may be operative in distinct inflammatory sites, even in a stereotyped phenomenon such as inflammatory leukocyte infiltration.

Recent research has highlighted a key role for IL-1β in gouty inflammation since IL-1β blockade abrogates inflammation [33, 34]. IL-1β is produced as an inactive pro-molecule by immune cells following immune stimulation and is then cleaved to its active form. The cleaving of pre-formed stores of pro-IL-1β into active IL-1β requires the assembly of a multi-protein complex called inflammasome [35]. In the case of gout, aberrant NLRP3 inflammasome activation is mediated by local

deposition of MSU crystals [36]. Our findings showed that the stimulation with MSU crystals increased the concentration of IL-1β in synovial fluid, suggesting that indeed there was activation of the inflammasome via MSU and consequent release of IL-1β. Of note, a recent study indicates that glibenclamide plays an inhibitory effect on inflammasome assembly by lipopolysaccharide and ATP *in vitro* [15]. In contrast with this *in vitro* study, our *in vivo* study demonstrated that glibenclamide treatment failed to prevent the increase in IL-1β levels induced by MSU injection, while the dexamethasone administration fully decreased it. Thus, the anti-nociceptive and anti-edematogenic effect of glibenclamide in MSU-treated animals does not seem to be dependent on the inhibition of the inflammasome. The discrepancy between the *in vitro* and the *in vivo* findings may be explained by the concentration of glibenclamide necessary to inhibit inflammasome *in vitro* is greater (> 50 μM) than the concentration of the drug found in tissues after *in vivo* administration. In fact, the maximal concentration of glibenclamide in plasma after *in vivo* administration is usually lower than 1 μM [37]. Moreover, in the cited *in vitro* study the inhibitory effect of glibenclamide on NLRP3 inflammasome activation was assessed by stimuli different from MSU crystals, additionally indicating that its inhibitory activity could be stimulus-dependent. Our results also have shown that glibenclamide can inhibit MSU-induced release in cultured macrophages, in a manner similar to that produced by lipopolysaccharide and ATP. However, it should be noted that the full inhibitory effect on IL-1 secretion was attained only at very high concentration (200 μM). We were precluded from increasing further the doses of glibenclamide in *in vivo* experiments due to its hypoglycemic effect. In any event and taking into account the plasma concentration of glibenclamide after oral administration [37], it is conceivable that the main anti-nociceptive effect of glibenclamide cannot be ascribed to its effect on

NLRP3 or IL-1β production, implying that other pathways must serve as target for glibenclamide.

One possibility to explain the potent anti-nociceptive effect of glibenclamide would be a putative effect on prostaglandin production. Prostaglandins play a pivotal role in the development of vasodilatation, fluid extravasation, and pain in synovial tissues and are found at elevated levels in the synovial fluid and membrane of arthritic patients [38]. In addition, inflammatory prostaglandins appear to play an especially prominent role in the onset of gout attacks since NSAIDs, which inhibit prostaglandin formation, are largely used for the treatment of acute gout [39]. Our results showed that the stimulation with MSU crystals indeed increased PGE_2 levels in the synovial fluid, but glibenclamide did not affect the release of PGE_2 whereas dexamethasone completely blocked it. Therefore, the anti-nociceptive and anti-edematogenic effects of glibenclamide in MSU-treated animals do not seem to be dependent on the release of prostaglandins. Of note, a recent study showed that silica crystals and aluminum salts, which are able to activate NLRP3 in a similar way of MSU can release PGE_2 [40], but this release is independent of inflammasome activation.

To confirm that the dose glibenclamide used here was capable of blocking K_{ATP} channels, we evaluated blood glucose levels in treated animals. We found that the administration of glibenclamide indeed reduced blood glucose levels, whereas dexamethasone increased blood glucose levels of MSU-injected animals. It has been demonstrated that glucocorticoids, such as dexamethasone, increases the levels of blood glucose by promoting gluconeogenesis in the liver [41]. On the other hand, the action of glibenclamide on blood glucose is in accordance with its action on pancreatic K_{ATP} channels, with release of insulin and reduction of glycaemia [42]. Of

note, insulin-releasing effect of glibenclamide may be related with its ability to reduce MSU-induced nociception since insulin may produce anti-nociception in rodents by a mechanism independent of glycaemia alterations [43]. However, considering that glibenclamide and dexamethasone shared a potent effect in inhibiting nociception and edema in our model in spite of having opposite effects on glycaemia, it is unlikely that glibenclamide effects on glycaemia may explain or interfere with its anti-nociceptive effects.

Taken together, the results presented herein indicate that glibenclamide reduced nociception and edema, but not leukocyte infiltration and IL-1β release in a model of acute gout attack. Its substantial potency (equivalent to dexamethasone) on nociception and edema suggests that glibenclamide can be an interesting option as an adjuvant treatment for pain induced by acute attacks of gout.

ACKNOWLEDGEMENTS

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FIGURES AND LEGENDS

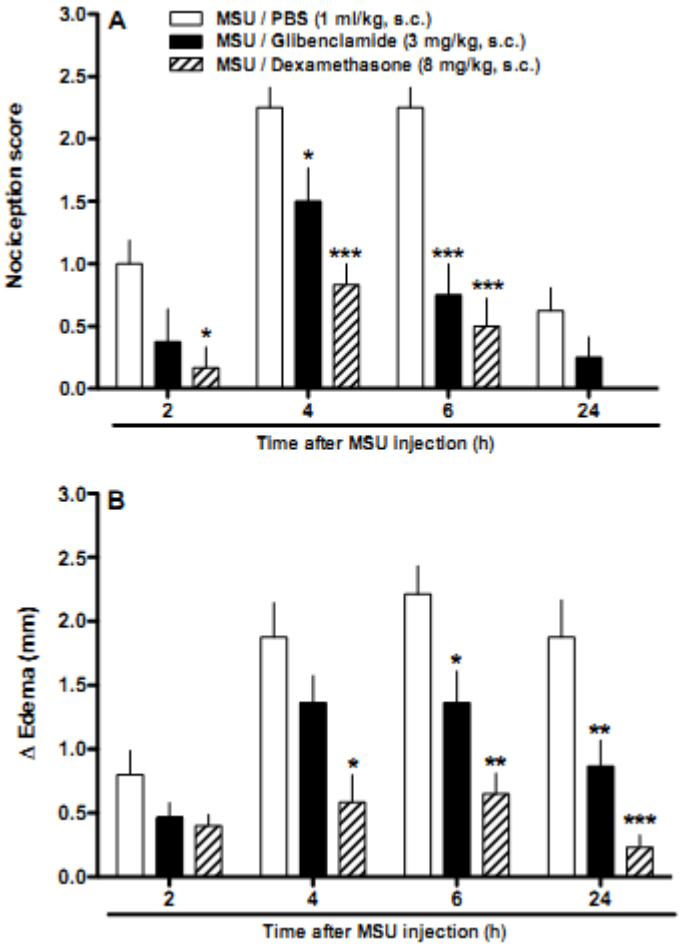

Fig 1 Time-course of the effect of the glibenclamide (3 mg/kg, s.c.) or dexamethasone (8 mg/kg, s.c.) on nociception (A) or edema (B) induced by intra-articular MSU injection (1.25 mg/site). The vertical bars represent the mean ± S.E.M. of six to eight animals per group. *P < 0.05; **P < 0.01 and ***P < 0.001 represent significant differences compared to vehicle-treated group; two-way ANOVA followed by Bonferroni test.

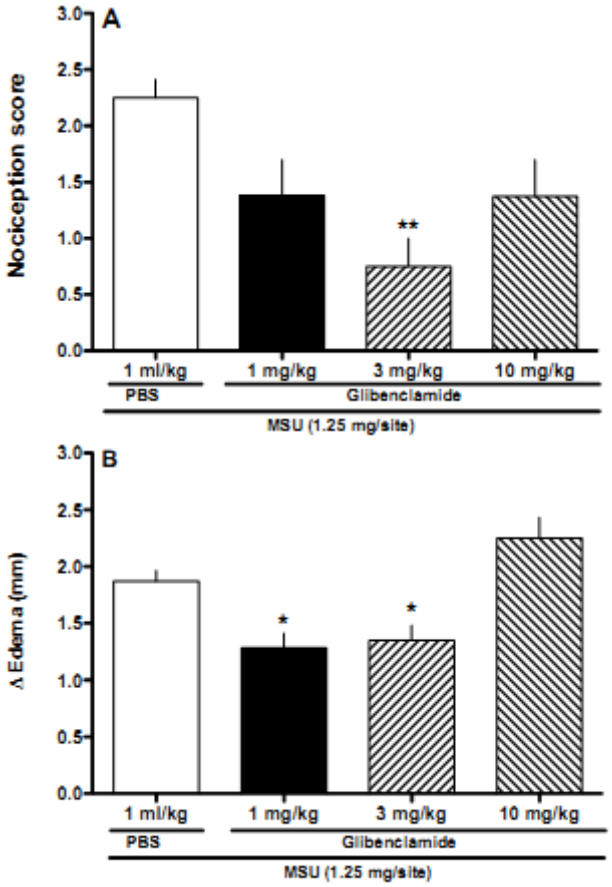

Fig 2 Dose-response curves for the anti-nociceptive effect (A) and of the anti-edematogenic effect (B) of glibenclamide (1-10 mg/kg, s.c.), 6 h after MSU (1.25 mg/site) administration. The vertical bars represent the mean ± S.E.M. of six to eight animals per group. *P < 0.05; **P < 0.01 represent significant differences compared to the vehicle-treated group. One-way ANOVA followed by Dunnett test.

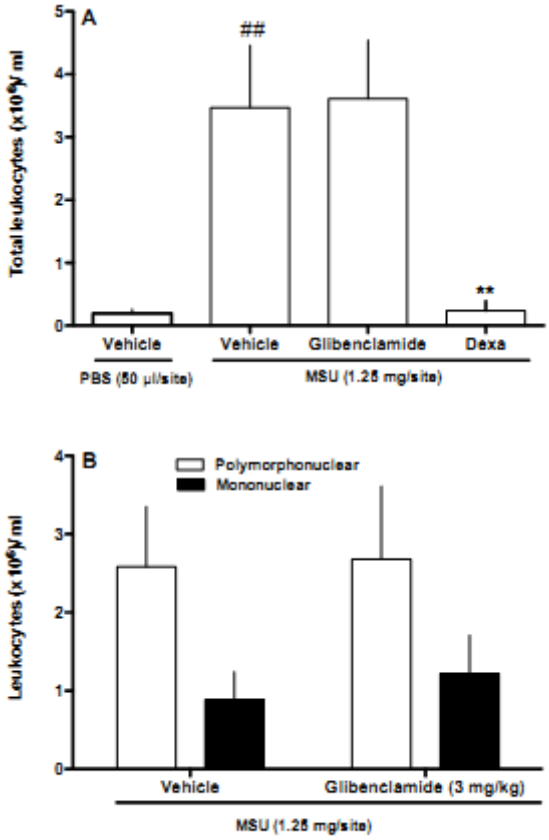

Fig 3 Effect of administration of glibenclamide or dexamethasone on the leukocyte infiltration induced by intra-articular MSU injection (1.25 mg/site). The animals were treated with vehicle, or dexamethasone (8 mg/kg) or glibenclamide (3 mg/kg, s.c.) and total cell counts (A) and differential counts (B) were determined 6 h after PBS or MSU intra-articular injection. The vertical bars represents the mean ± S.E.M. of eight animals by group; ##P<0.01 when compared to vehicle plus PBS group; **P < 0.001 when compared to vehicle plus MSU group. One-way ANOVA followed by Dunnett test.

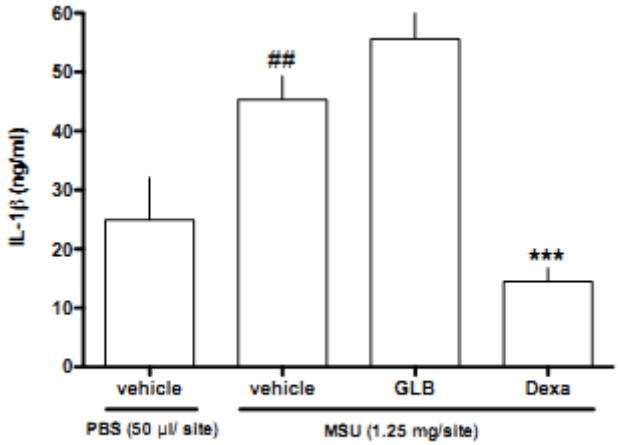

Fig 4 Effect of administration of glibenclamide (GLB) or dexamethasone (Dexa) on IL-1β levels induced by intra-articular MSU injection (1.25 mg/site). The animals were treated with vehicle, or dexamethasone (8 mg/kg) or GLB (3 mg/kg, s.c.) and assayed for cytokine levels, 6 h after PBS or MSU intra-articular injection. The vertical bars represents the mean ± S.E.M. of six animals by group; ## P < 0.01 when compared to vehicle plus PBS group; ***P < 0.001 when compared to vehicle plus MSU group; One-way ANOVA followed by Dunnett test.

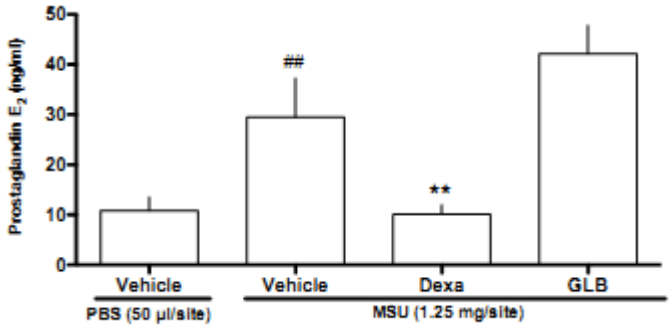

Fig 5 Effect of administration of glibenclamide or dexamethasone on prostaglandin E_2 levels induced by intra-articular MSU injection (1.25 mg/site). The animals were treated with vehicle, or dexamethasone (8 mg/kg) or glibenclamide (3 mg/kg, s.c.) and assayed for prostaglandin E_2, 6 h after PBS or MSU intra-articular injection. The vertical bars represents the mean ± S.E.M. of five animals by group; $^{\#\#}$ P < 0.01 when compared to vehicle plus PBS group; **P < 0.01 when compared to vehicle plus MSU group; One-way ANOVA followed by Dunnett test.

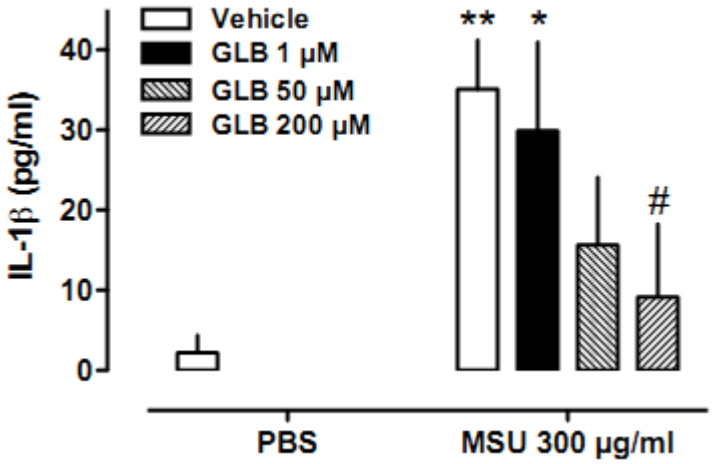

Fig 6 Interleukin-1β release by mouse peritoneal macrophages activated with MSU. Glibenclamide was added 30 min after MSU (300 µg/mL) and 24 h later, IL-1β was assayed in the supernatant. Vertical bar represents the mean ± S.E.M of three animals by group. **P < 0.01 and *P < 0.05 compared to vehicle group; # P < 0.05 compared to vehicle plus MSU group; two-way ANOVA followed by Bonferroni's post hoc t-test.

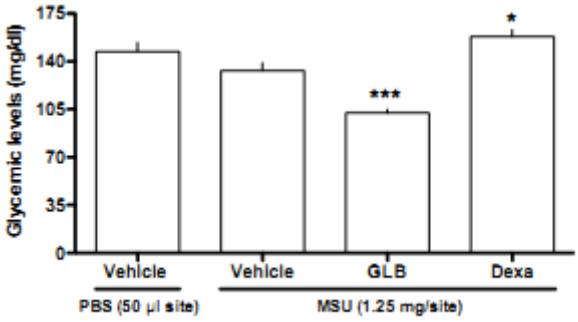

Fig 7 Effect of the glibenclamide (GLB) or dexamethasone (Dexa) on blood glucose levels of rats that received intra-articular MSU injection (1.25 mg/site). Blood glucose levels of animals treated with vehicle (1 ml/kg), GLB (3 mg/kg, s.c.) or dexamethasone (8 mg/kg, s.c.) at 6 h of PBS or MSU injection. Vertical bars represent the mean ± S.E.M. of four to six animals. ***P < 0.001 when compared to MSU/vehicle group; one-way ANOVA followed by Dunnett test.

5. CONCLUSÕES

Com base nos resultados obtidos no presente estudo, pode-se concluir que:

5.1. A glibenclamida apresentou efeitos anti-nociceptivo e anti-edematogênico em modelo de gota aguda induzida por MSU;

5.2. A glibenclamida não apresentou efeitos sobre a infiltração de leucócitos e liberação de IL-1β no líquido sinovial de ratos em modelo de gota aguda, induzida por MSU;

5.3. A glibenclamida na concentração de 200 μM foi capaz de reduzir a liberação de IL-1β induzida por MSU, em cultura de macrófagos;

5.4. A glibenclamida não apresentou efeito na produção de prostaglandina E_2 em modelo de gota aguda, induzida por MSU;

5.5. Os efeitos apresentados pela glibenclamida parecem não estar relacionados à via do inflamassoma ativada pelo MSU;

5.6. Os efeitos desempenhados pela glibenclamida parecem não estar associados com a liberação de prostaglandinas induzida pelo MSU.

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7. APÊNDICE

7.1. **Apêndice A –** Manuscrito em preparação: Effects of tetraethylammonium on inflammatory parameters induced by monosodium urate crystals in rats

EFFECTS OF TETRAETHYLAMMONIUM ON INFLAMMATORY PARAMETERS INDUCED BY MONOSODIUM URATE CRYSTALS IN RATS

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ABSTRACT

Objective: We investigated the effect of TEA (tetraethylammonium), a non-selective blocker of potassium channels, on inflammatory parameters in a model of acute gouty attack in rats.

Treatment: Intra-articular injection of 50 µl of monosodium urate (MSU) crystals (1.25 mg/site) was used to induce gout-related inflammation. The effects of TEA (8-16 mg/kg, s.c.) or dexamethasone (8 mg/kg, s.c., positive control) were assessed on several inflammation parameters.

Methods: Spontaneous nociception, articular edema, total leucocyte counts and interleukin (IL)-1β release were analyzed. Statistical significance was assessed by one- or two-way analysis of variance.

Results: TEA (8-16 mg/kg) did not decrease nociception and edema induced by MSU injection in rats, in contrary dexamethasone (8 mg/kg) prevented these parameters. The treatment with TEA (16 mg/kg) or dexamethasone significantly reduced leukocyte infiltration and IL-1β release induced by MSU. NS1619 (an activator of high conductance calcium-activated potassium channels) treatment neither altered inflammatory parameters per se nor prevented the effects of TEA.

Conclusions: TEA reduced leukocyte infiltration and IL-1β release, but not nociception and edema induced by articular MSU, effects that appear not to be dependent of blockade on calcium-activated potassium channels.

Key words: nociception; edema; leukocyte; potassium channel; interleukin-1β.

1. INTRODUCTION

Gout is a progressive rheumatic disease caused by the cumulative deposition of monosodium urate crystals (MSU) within the soft tissue and joints. Acute gout attacks typically presents as painful, intermittent flares of severe joint inflammation. Research has highlighted a key role for IL-1β in gouty inflammation whereby the blockade of IL-1β abrogates inflammation (Chen et al., 2006). Although gout is a potentially treatable disease, its diagnosis and treatment are poorly managed worldwide (Rott and Agudelo, 2003).

Studies have shown that potassium channels (KC) play an important role in intracellular signaling cascades inflammatory (Czaika et al., 2000) and that high concentrations of K^+ prevents the release of IL-1β (Perregaux et al. 1994; Walev et al. 1995; Cheneval, et al. 1998; Kahlenberg and Dubyak, 2004). Moreover, KC can inhibit the activation of macrophages (Maruyama et al., 1994; Walev et al., 1995) resulting in the reduced production of cytokines (Blunck et al., 2001; Papavlassopoulos et al. 2006). These data allow us to speculate that the role of KC in inflammatory reactions may also occur during the onset of gouty arthritis, and these effects can also be modulated by blockers or openers of these channels. We previously showed that there is involvement of ATP-sensitive KC (K_{ATP} channels) on nociception and edema induced MSU, since that glibenclamide, a blocker of K_{ATP} channels, reduced nociception and edema, but not leukocyte infiltration and IL-1β release in a model of acute gout attack. However, the role of other subtypes of KC has not been elucidated.

Thus, this study was designed to evaluate the participation of a nonselective KC blocker (TEA) and an activator of high conductance calcium-activated KC (NS1619)

in the inflammatory effects triggered by intra-articular MSU in a model of acute gout attack.

2. MATERIALS AND METHODS

2.1. *Animals*

Male Wistar rats weighing 250-300 g were housed in a temperature- and light-controlled room (22±1 °C with 12 h light/dark cycle), with free access to water and food. The animals were acclimated to the experimental room for at least 1 hour before the experiments. All animal care and experimental procedures were approved by the University Institutional Ethics Committee (process number 23081.003640/2009-61) and are in accordance with CONCEA (National Council for the Control of Animal Experimentation) Guidelines.

2.2. *Drugs and reagents*

Tetraethylammonium (TEA), a non-selective blocker of potassium channels) and NS1619 (an activator of high conductance calcium-activated potassium channels) were obtained from Sigma Chemical® (St. Louis, USA) and dexamethasone was purchased from Aché® (São Paulo, Brazil).

Synthetic monosodium urate (MSU) crystals were prepared as described previously (Hoffmeister et al., 2011) and suspended in phosphate-buffered saline (PBS; 10.71 mM K_2HPO_4, 6.78 mM NaH_2PO_4, 120.4 mM NaCl; pH 7.4). Polarized light microscopic examination confirmed that the crystals were rod-shaped and varied in length (12 ± 2 μm). The preparation was endotoxin free as determined by an amebocyte cell lysate assay (Sigma®, St Louis, USA).

2.3. *MSU crystals-induced arthritic gout and therapeutic groups*

The experiments were conducted in accordance with the method described by Coderre and Wall (1988), with some modifications. Briefly, we injected 50 µL of vehicle (PBS) or MSU suspension (1.25 mg/site) intra-articularly (i.a.) into the tibio-tarsal joint (ankle) on isoflurane-anesthetized animals. To investigate the possible involvement of potassium channels in MSU- induced arthritic gout, the opener of high conductance calcium-activated potassium channels NS1619 (30 µg/site) was co-injected with MSU (1.25 mg/site, i.a.) and the non-selective blocker of potassium channels TEA (8 - 16 mg/kg) was administered subcutaneously (s.c.), immediately after crystal injection. The involvement of the TEA on NS1619 was investigated; to elucidate its participation, the TEA was coadministered (s.c.) with NS1619 + MSU (1.25 mg/site, i.a.) before of the measurements of inflammatory and nociceptive parameters. As a positive control, we also evaluated the effects of dexamethasone (8 mg/kg, s.c.) on nociception and edema induced by MSU. The doses of treatment were based in previous study (Sordi et al, 2011).

2.4. *Evaluation of nociception*

The spontaneous nociception was assessed using a standing paw pressure score from 0 until 3, as previously described by Coderre and Wall (1988). Rats were habituated for 20 minutes to observation in glasses box and the paw pressure score was determined. The amount of weight that the rat was willing to put on the hindpaw of the injected limb was evaluated and categorized according to the scale, as described below: score 0 was considered when the paw pressure was normal, with equal weight on both hind-paws; score 1 was considered when the paw pressure was slightly reduced, with the paw completely on the floor, but toes are not spread;

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 foot elevated completely.

2.5. Measurement of edema

Animals were individually placed in transparent glass chambers and allowed to adapt to their surroundings for 20 minutes and the edema was measured. The edema formation was assessed as an increase in ankle thickness, in millimeter (mm), after MSU injection with a digital caliper (Hoffmeister et al., 2011), when compared to baseline values. Results were expressed as variation of edema (difference between the measurement of ankle thickness before and after treatment).

2.6. *Collection of synovial fluid, leucocyte count and IL-1β determination*

Six hours after MSU injection, animals were anaesthetized with sodium pentobarbital (100 mg/kg, i.p.) and perfused to avoid blood interference. Injected ankle synovial cavities were washed with 60 µL of PBS (three times of 20 µL each) by inserting a tip into the rat ankle joint, and the synovial washes were recovered by aspiration (Conte et al. 2010). To determine whether the treatment with TEA, NS1619 or TEA plus NS1619 induced changes in leukocyte numbers, synovial fluid samples from animals treated with vehicle (1 mg/ml, s.c.), TEA (16 mg/kg, s.c.), NS1619 (30 µg/site), TEA (16 mg/kg) + SD 1619 (30 µg/site) or dexamethasone (8 mg/kg, s.c.) were subjected to total leukocyte count and IL-1β determination.

The concentrations of the IL-1β were quantified by an enzyme-linked immunosorbent assay (ELISA) kit, according to the manufacturer's instructions

(PeproTech®, Rocky Hill, USA). Total leucocyte count were performed in a Neubauer chamber after dilution of the synovial fluid samples in Turk's solution (0.01% Gentian violet and 1% glacial acetic acid in distilled water) under an optical microscope and values are expressed as numbers of cells per milliliter ($\times 10^6$).

2.8. *Statistical analysis*

All values are expressed as mean ± S.E.M. The statistical significance between groups was assessed by one- or two-way analysis of variance (ANOVA) when appropriate. Pos-hoc tests (Dunnett's test for one-way or Bonferroni for two-way ANOVA) were also carried out when appropriate. P values lower than 0.05 ($P<0.05$) were considered to be indicative of significance.

3. RESULTS

3.1. *Participation of TEA* and SD 1619 on *MSU-induced nociception and edema induced by MSU*

The i.a. injection of MSU crystals (1.25 mg/paw) caused nociception and edema when injected to the rat ankle joint (Fig. 1A and B). Treatment with TEA (8 or 16 mg/kg, s.c., a non-selective KC blocker) was unable of reduce the spontaneous nociception and the edema induced by MSU; differently, the treatment with dexamethasone (8 mg/kg, s.c., used as a positive control) was able to reduce the spontaneous nociception and the edema MSU-induced (Figure 2A and B).

When NS1619 (30 µg/site, an opener of high conductance calcium-activated potassium channel) was co-administrated with MSU (1.25 mg/site), it was not able to change MSU-induced nociception and edema. Likewise, NS1619 (30 µg/site) plus

TEA (16 mg/kg) was also unable to alter the nociception or the edema trigged by MSU (Fig. 4A and B).

3.2. *Participation of TEA and NS1619 on leukocytes infiltration induced by MSU*

The synovial fluid of the ankle joint was collected and the accumulation of inflammatory cells was determined. The number of infiltrating cells was substantially increased after 6 h of MSU crystal injection (Fig. 3A). TEA (16 mg/kg, s.c.) or dexamethasone (8 mg/kg, s.c.) administration were able to significantly reduce the total leukocyte infiltration (73±13% and 94±6% of reduction, respectively) (Fig. 3A).

We also verify the action of NS1619 on leukocytes infiltration induced by MSU in the synovial cavity. Co-administration of the NS1619 (30 µg/site) plus MSU (1.25 mg/site) did not alter the effects of MSU on leukocytes infiltration in the synovial cavity (Fig. 4C). Moreover, when animals were treated with TEA and received co-administration of MSU (1.25 mg/site) plus NS1619 (30 µg/site), the cells count was not significantly different compared to treatment with TEA alone (Fig. 4C).

3.2. *Participation of TEA on release IL-1β induced by MSU*

We observe in the present study that the stimulation with MSU crystals enhanced the concentration of IL-1β in synovial fluid (Fig. 3B). TEA (16 mg/kg, s.c.) treatment was able to prevent the increase of IL-1β induced by MSU injection, as well as the dexamethasone (8 mg/kg, s.c.) administration fully inhibited MSU-induced IL-1β release (52±15% and 100% of inhibition, respectively).

DISCUSSION

The worldwide incidence and prevalence of gout have been increasing over the last few decades, making gout far and away the most common inflammatory arthritis. Gout affects at least 1% of the population in developed countries (Smith et al., 2010). This complex condition and the high prevalence call for more pharmacological options (Kingsbury et al., 2011).

MSU stimulation of the inflammatory cascade is initiated by the activation of articular resident mononuclear phagocytes, leading to a host of pro-inflammatory mediators such as IL-1β (Di Giovine et al., 1987). Evidence derived from both experimental and clinical studies indicates IL-1β as a major player in gout (Torres et al., 2009; So et al., 2007). This cytokine is produced as an inactive proform that must be cleaved within the cell to generate biologically active IL-1β. The enzyme caspase-1 catalyzes the reaction (Petrilli and Martinon, 2007). Results of the present study demonstrated that the MSU increased the release of IL-1β in the synovial fluid, and administration of TEA (16 mg/kg, s.c.) reverts it (Figure 3B). TEA blocks KC, increasing the intracellular concentration of K^+. In line with this, studies have shown that high concentrations of K^+ prevents the release of IL-1β, suggesting that the reduction in the intracellular concentration of K^+ is required for the processing of IL-1β, through activation of caspase-1 enzyme (Perregaux et al., 1994; Walev et al., 1995; Cheneval et al., 1998; Kahlenberg and Dubyak, 2004). In addition, as a potent pro-inflammatory cytokine, IL-1β affects every cell type and promotes infusion of leukocytes (Chen et al., 2006); our data are consistent with this, since that we observed that MSU produced an extensive leukocyte infiltration in the synovial fluid of rats. The TEA treatment (16 mg/kg, s.c.) also decreases the leukocyte migration (Figure 3A), suggesting that the effect of TEA on the flow of K^+ across cells influence the release of IL-1β and it would act on the influx of leukocytes into synovial fluid. We

previously demonstrated that a blocker of K_{ATP} channels (glibenclamide) was able to reduce nociception and edema, but not infiltration of leukocytes and release of IL-1β in this same model of acute attack of gout. Thus, we suggest that the effects of TEA shown here might be due to inhibition of others KC, but the K_{ATP} channels. TEA inhibits preferentially calcium-activated KC, whereas at higher doses, it inhibits most, if not all, KC subtypes (Nelson and Quayle, 1995). In accordance with this, a prior study of our group (Sordi et al., 2011) indicates that selective blockade of voltage-gated and ATP-sensitive KC does not reproduce the beneficial effects performed for TEA in model of sepsis, suggesting that the calcium-activated KC are the more likely sites of action of TEA. Thus, with the purpose of check the involvement of calcium-activated KC in the effects played by TEA in this study, we decided to use an opener calcium-activated KC, the compound NS1619. Activation of this KC subtype did not induce any effects, either protective or harmful the TEA effects, on inflammatory parameters induced by MSU in rats. Thereby, in this model of acute gout induced by MSU, seems that the effects of TEA are not dependent on the KC activate to calcium or ATP-sensitive KC.

Gouty arthritis is characterized by early vasodilation, enhanced vascular permeability, erythema and pain (Busso, 2010).Our results showed that rats injected with MSU (i.a.), but not with PBS, developed edema and nociception, with higher intensity at 4 to 6h after MSU administration (Figure 1A and B). In our hands, TEA (8 or 16 mg/kg, s.c.) did not affect the increase in the nociception and edema induced by MSU.

In summary, the results presented herein indicate that TEA reduced leukocyte infiltration and IL-1β release, but not nociception and edema in a model of acute gout attack. Accordingly, KC could be mediating by these responses. Thus, further studies

should be conducted to clarify the actions of TEA on the gout, and the possible involvement of KC in the evolution of inflammatory responses triggered in gouty arthritis.

Figure for Legends

Figure 1: Time-course of the effect of the TEA (16 mg/kg, s.c.) or dexamethasone (8 mg/kg, s.c.) on nociception (A) or edema (B) induced by intra-articular MSU injection (1.25 mg/site). The vertical bars represent the mean ± S.E.M. of six animals per group. *P < 0.05 and **P < 0.01 represent significant differences compared to vehicle-treated group; two-way ANOVA followed by Bonferroni test.

Figure 2: Dose-response curves for the anti-nociceptive effect (A) and of the anti-edematogenic effect (B) of TEA (8 or 16 mg/kg, s.c.), 6 h after MSU (1.25 mg/site) administration. The vertical bars represent the mean ± S.E.M. of six to eight animals per group. **P < 0.01; ***P < 0.001 represent significant differences compared to the vehicle-treated group; one-way ANOVA followed by Dunnett test.

Figure 3: Effect of administration of TEA or dexamethasone on the leukocyte infiltration (A) and on IL-1β levels (B) induced by intra-articular MSU injection (1.25 mg/site). The animals were treated with vehicle, dexamethasone (8 mg/kg) or TEA (16 mg/kg, s.c.) and total cell counts and cytokine levels were determined 6 h after PBS or MSU intra-articular injection. The vertical bars represents the mean ± S.E.M. of eight animals per group; $^{\#\#}$P<0.01 when compared to vehicle plus PBS group; *P < 0.05 and **P < 0.01 when compared to vehicle plus MSU group; one-way ANOVA followed by Dunnett test.

Figure 4: Effect of the TEA (16 mg/kg, s.c.), NS1619 (30 µg/site, i.a.) or TEA (16 mg/kg s.c.) plus NS1619 (30 µg/site i.a.) on nociception (A) or edema (B) or leukocyte infiltration (C) induced by intra-articular MSU injection (1.25 mg/site). The animals were treated with vehicle, TEA (16 mg/kg, s.c.), NS1619 (30 µg/site, i.a.) or SD 1619 (30 µg/site, i.a.) plus TEA (16 mg/kg, s.c.) and inflammatory parameters was determined 6 h after PBS or MSU intra-articular injection. The vertical bars represents the mean ± S.E.M. of six to eight animals by group; *P < 0.05 when compared to vehicle plus MSU group; one-way ANOVA followed by Dunnett test.

Figures

Figure 1

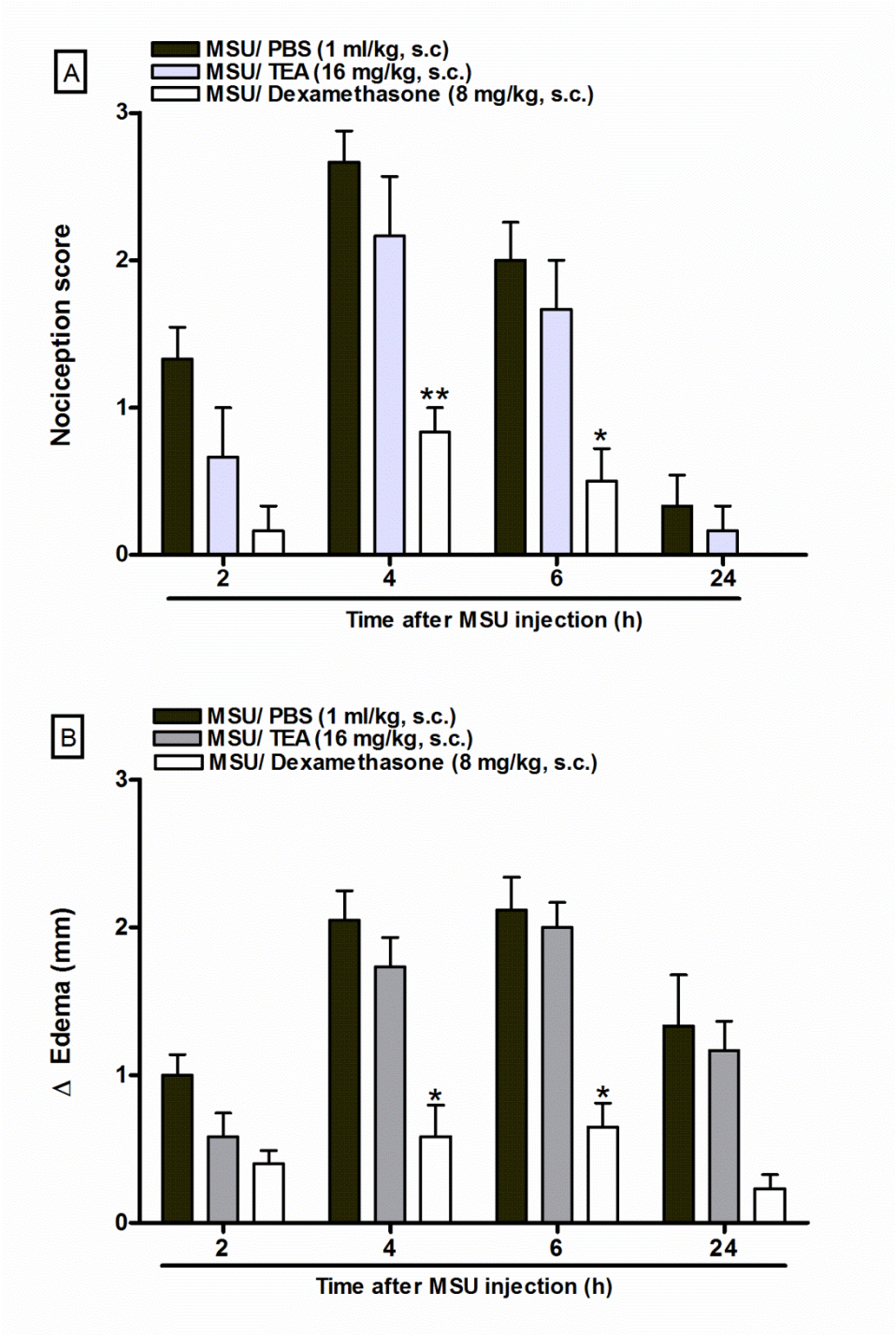


Figure 2

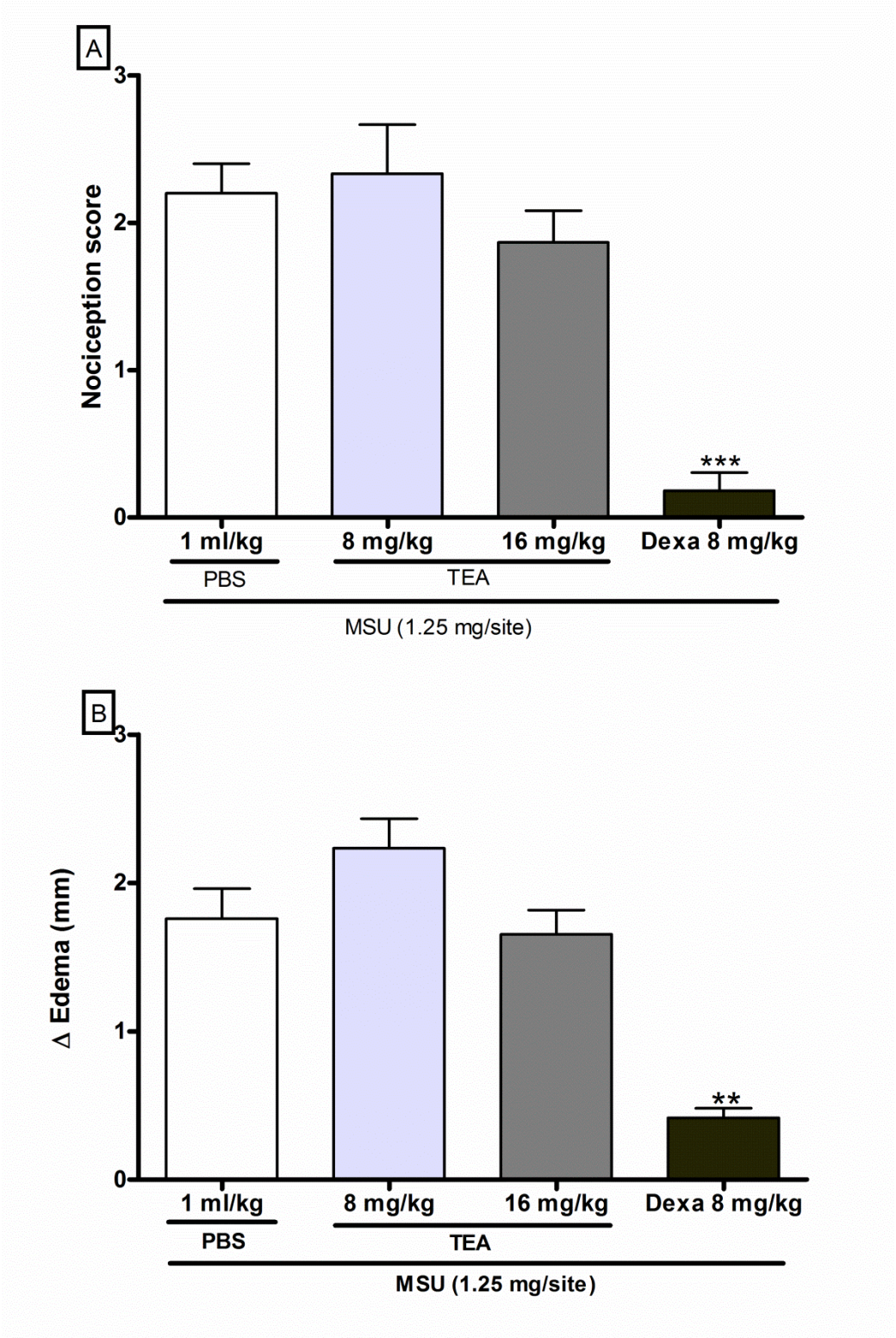

A
Nociception score
3
2
1
0
***
1 ml/kg
8 mg/kg
16 mg/kg
Dexa 8 mg/kg
PBS
TEA
MSU (1.25 mg/site)
B
Δ Edema (mm)
3
2
1
0
**
1 ml/kg
8 mg/kg
16 mg/kg
Dexa 8 mg/kg
PBS
TEA
MSU (1.25 mg/site)

Figure 3

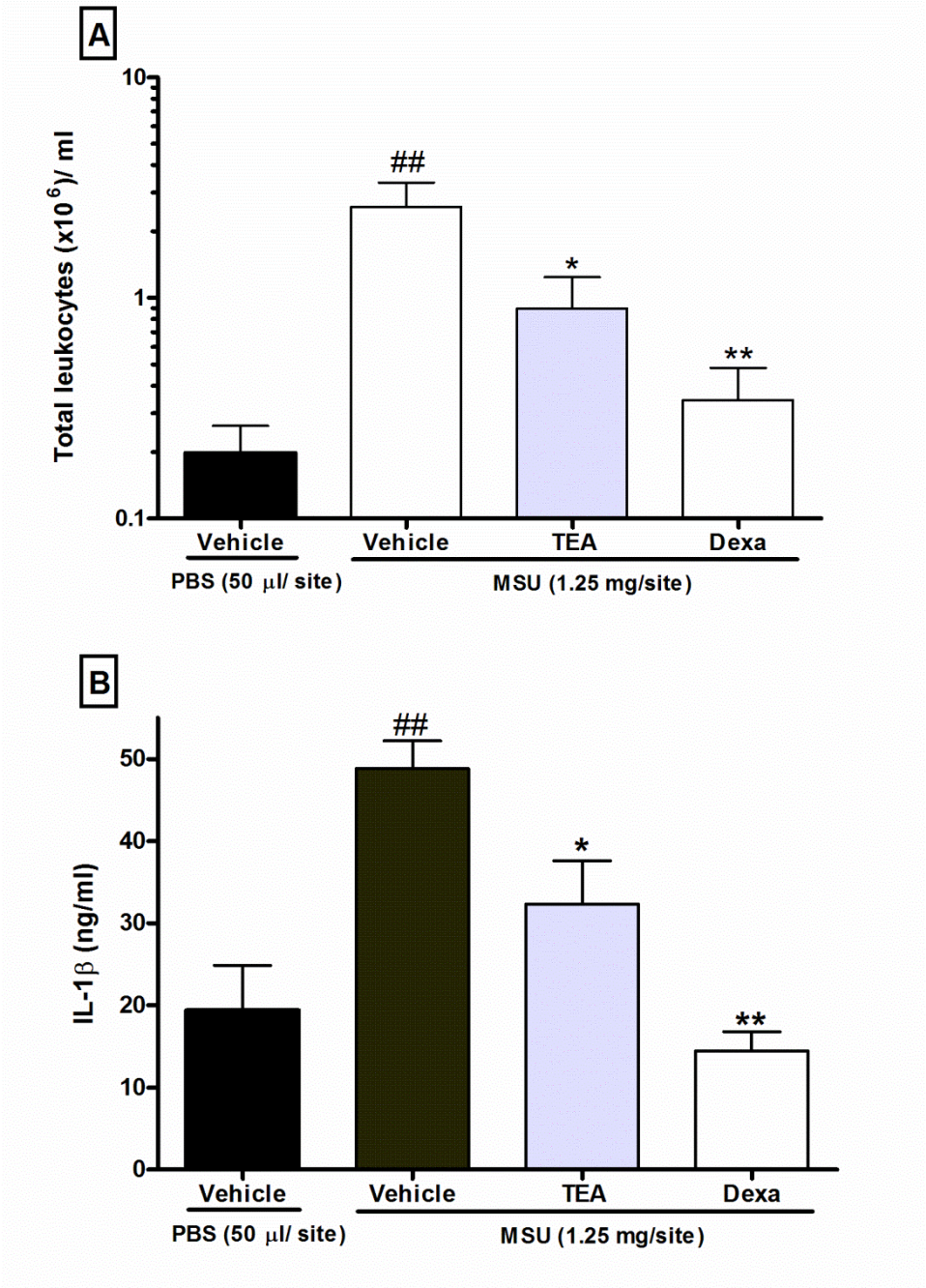
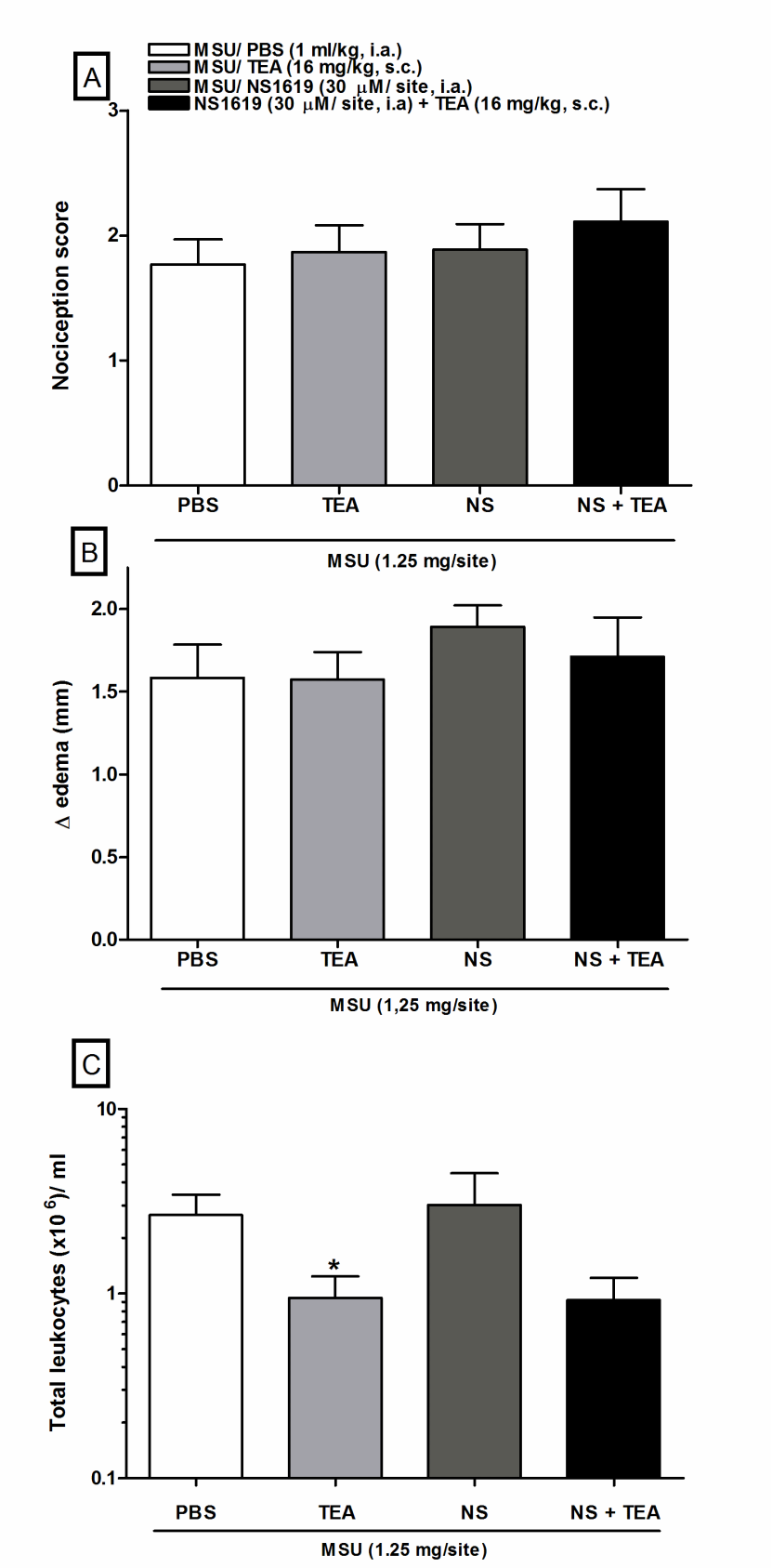

A
Total leukocytes (x10 6)/ ml
10
1
0.1
##
*
**
Vehicle
Vehicle
TEA
Dexa
PBS (50 µl/ site)
MSU (1.25 mg/site)
B
IL-1β (ng/ml)
50
40
30
20
10
0
##
*
**
Vehicle
Vehicle
TEA
Dexa
PBS (50 µl/ site)
MSU (1.25 mg/site)

Figure 4


A
MSU/ PBS (1 ml/kg, i.a.)
MSU/ TEA (16 mg/kg, s.c.)
MSU/ NS1619 (30 μM/ site, i.a.)
NS1619 (30 μM/ site, i.a) + TEA (16 mg/kg, s.c.)
Nociception score
3
2
1
0
PBS
TEA
NS
NS + TEA
MSU (1.25 mg/site)
B
Δ edema (mm)
2.0
1.5
1.0
0.5
0.0
PBS
TEA
NS
NS + TEA
MSU (1,25 mg/site)
C
Total leukocytes (x10 6)/ ml
10
1
0.1
*
PBS
TEA
NS
NS + TEA
MSU (1.25 mg/site)

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