IVAN JOSÉ MAGAYEWSKI BONET

"O papel do receptor TRPA1 no desenvolvimento e manutenção da hiperalgesia induzida pela carragenina"

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Dissertação apresentada ao Instituto de Biologia para obtenção do Título de Mestre em Biologia Funcional e Molecular, na área de Fisiologia.

Orientadora: Profa. Dra. Claudia Herrera Tambeli

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“Não desejes e serás o homem mais rico do mundo.”

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RESUMO

O Receptor Potencial Transiente Ankiryn 1 (TRPA1) é um canal não seletivo a cátions importante na fixação do limiar nociceptivos e pertencente a superfamília de canais TRP. É expresso em fibra C-nociceptiva e células não neuronais envolvidas na liberação de mediadores pró-inflamatórios. No presente estudo, investigamos se o TRPA1 contribui para a hiperalgesia induzida pela carragenina em ratos, e se essa contribuição é mediada por mecanismos de inflamação, tais como liberação de citocinas pró-inflamatórias e migração de neutrófilos e/ou sensibilização direta do neurônio aferente primário. Avaliamos a sensibilização do nociceptor induzida pela carragenina utilizando estímulos mecânico (analgesímetro mecânico) e químico (capsaicina), com ou sem bloqueio farmacológico local do receptor TRPA1 pelo seu antagonista seletivo HC 030031. A carragenina induziu hiperalgesia com pico na terceira hora, persistindo até vigésima quarta hora. O bloqueio farmacológico do receptor TRPA1 por co-administração de HC 030031 diminuiu significativamente a hiperalgesia induzida pela carragenina na terceira hora e a pós-administração de HC 030031 (2hrs 55min) reduziu na terceira e na sexta hora. O silenciamento do gene do TRPA1, induzido por um pré-tratamento intratecal com Oligonucleotídeo antisense, preveniu a hiperalgesia induzida pela carragenina após 24 horas além de reduzir significativamente a expressão de TRPA1 em células dos gânglios da raiz dorsal (GRD) (L5-6). O tratamento com carragenina, por sua vez, não alterou a expressão do receptor TRPA1 no GRD, e tampouco afetou os níveis de citocinas e a migração de neutrófilos no tecido periférico (patas). Concluímos que TRPA1 tem papel importante no

desenvolvimento e manutenção da hiperalgesia inflamatória induzida pela carragenina por contribuir diretamente na excitabilidade do nociceptor. Baseado nesses achados, sugerimos que o bloqueio de TRPA1 é uma estratégia promissora no desenvolvimento de futuras drogas para o controle e tratamento da dor.

ABSTRACT

The Transient Receptor Potential Ankiryn 1 (TRPA1) is a nonselective cation channel, important in setting nociceptive threshold belonging to the superfamily of TRP channels. It is expressed in nociceptive C-fibers and in non-neuronal cells involved in pro-inflammatory mediators release. In this study, We asked whether TRPA1 contributes to carrageenan-induced hyperalgesia in rats, and whether this contribution is mediated by mechanisms in inflammation, such as cytokine release and neutrophil migration and/or by a direct sensitization of the primary afferent nociceptors. We measured the carrageenan-induced nociceptive sensitization using a mechanical (mechanical analgesymeter) and a chemical (capsaicin) stimulus, with or without pharmacological blockade of TRPA1 by its selective antagonist HC 030031. Carrageenan-induced Hiperalgesia has peaked at the third hour and persisted until the twenty-fourth hour. Pharmacological blockade of TRPA1 receptor by co-administration of HC 030031 significantly lowered carrageenan-induced hiperalgesia at the third hour and post-administration (2hrs 55min) decreased at both third and sixth hours. The neuronal TRPA1 gene silencing induced by intrathecal pre-treatment with antisense oligodoexynucleotide completely prevented carrageenan-induced hyperalgesia over 24 hours and significantly reduced TRPA1 expression in the dorsal root ganglia cells (DRG) (L5-6). However, carrageenan treatment, did not affect the TRPA1 expression on DRG, neither affected the cytokines levels and neutrophil migration in peripheral tissue (paws). We conclude that TRPA1 plays an important role in the development and maintenance of carrageenan-induced inflammatory hyperalgesia by directly contributing to

nociceptor excitability. Based on these findings, we suggest that TRPA1 blockade is a promising strategy for the development of future drugs to pain treatment and control.

SUMÁRIO

I. INTRODUÇÃO

A dor é considerada um dos maiores problemas de saúde pública da sociedade atual e embora nosso conhecimento sobre os mecanismos nociceptivos tenha evoluído muito nos últimos anos, os fármacos utilizados para o controle da dor hoje pertencem à mesma classe de fármacos utilizados há décadas. São basicamente anti-inflamatórios e analgésicos opióides cujo uso clínico é marcado por um alto índice de insucessos e/ou de efeitos colaterais que se intensificam com o uso crônico. A maioria das condições dolorosas é de origem inflamatória periférica e vários estudos experimentais têm sido conduzidos com o objetivo de identificar novos alvos moleculares para o tratamento da dor inflamatória em sua origem, ou seja, na periferia. De acordo com a Associação Internacional de Estudos da Dor a dor é definida por "uma experiência sensorial e emocional desagradável associada a um dano tecidual real ou potencial, ou descrita em termos de tal dano".

A. Nocicepção

De um ponto de vista evolutivo, a percepção dolorosa, se refere a um comportamento aversivo a uma sensação desagradável, e foi um dos principais determinantes à sobrevivência das espécies. Um estímulo, tanto interno quanto externo, é detectado por estruturas receptoras localizadas por todo o corpo denominadas nociceptores. Os nociceptores fazem a transdução do estímulo nociceptivo transformando-o em um impulso nociceptivo que é conduzido até o

sistema nervoso central (SNC) por meio das fibras aferentes primárias, que se comunicam com neurônios de segunda ordem, os quais conduzem os impulsos nociceptivos até os centros superiores para que sejam processados (BONICA, 1990). Por esse prisma, a dor tem um importante papel de proteção dos tecidos orgânicos.

A terminologia nociva se refere a algo que causa dano, podendo ser um estímulo que cause algum dano a um determinado tecido, ou seja, algo prejudicial ao organismo. Já o termo nociceptivo refere-se a algo que ativa as vias sensoriais especializadas em conduzir a informação produzida por esses estímulos nocivos ou potencialmente nocivos. Então, um estímulo nociceptivo tem como característica, ser capaz de ativar essas vias. Essas vias, porém, nem sempre são ativadas somente por estímulos nocivos. Elas podem ser ativadas por estímulos que não causam dano ao organismo, mas que possuem intensidade suficiente para ativá-las. Podemos, de forma simplista, descrever a dor como uma espécie de alarme fisiológico, alertando o organismo de algo possivelmente danoso.

As vias de condução da informação nociceptiva da região de tronco e membros são compostas por conjuntos de neurônios periféricos (fibras aferentes primárias) e por conjuntos de neurônios medulares (neurônios de segunda ordem) (AGUGGIA, 2003). As fibras aferentes primárias são neurônios pseudounipolares, cujos corpos celulares estão localizados no gânglio da raiz dorsal (DRG) e emitem ramificações para periferia e para o corno dorsal da medula espinal (AGUGGIA, 2003). As fibras nociceptivas, por possuírem alto limiar de ativação e terminações

nervosas livres (não associadas a receptores sensoriais especializados), estão diretamente relacionadas a estímulos intensos variados. Existem dois tipos de fibras que conduzem estímulos nociceptivos. Fibras A-delta (Aδ), de diâmetro médio são finamente mielinizadas, e por essas variáveis fisiológicas, possuem velocidade de condução média, entre 12 e 30 m/s. Elas correspondem a 20% das fibras de dor e são responsáveis pela condução da informação referente à dor rápida, aguda e lancinante que é sentida após estimulação nociva. Já as fibras C não-mielinizadas, de pequeno diâmetro, possuem uma velocidade de condução menor (0,5 a 2 m/s) e correspondem a 80% das fibras condutoras da dor sendo responsáveis pela condução da informação referente à dor lenta e difusa (JULIUS; BASBAUM, 2001). As fibras do tipo Aδ respondem a estimulações de origem mecânica, porém podem ser sensibilizadas por estímulo térmico, enquanto as fibras do tipo C respondem a estímulos mecânico, térmico e também químico, sendo denominadas de nociceptores polimodais.

A nocicepção basicamente é um fenômeno iônico, através do qual um estímulo capaz de ativar os nociceptores é detectado e conduzido por neurônios da via nociceptiva. A natureza do estímulo pode ser mecânica, térmica ou química e sua origem pode ser interna ou externa ao organismo (AGUGGIA, 2003). Quando o estímulo alcança o limiar de disparo dos potenciais de ação das fibras aferentes primárias, um impulso elétrico é gerado e conduzido por estas fibras até os centros superiores onde são processados e percebidos como dor (AGUGGIA, 2003). Assim, a ativação dos nociceptores é capaz de deflagrar um potencial de ação que é transmitido ao longo das fibras aferentes primárias até a medula

espinal, onde ocorrem sinapses com o neurônio de segunda ordem, que por sua vez enviam projeções ascendentes conduzindo a informação nociceptiva a sítios intermediários ou diretamente ao tálamo. Uma via específica de condução se projeta do tálamo para áreas sensitivas do córtex, enquanto outra também projetada do tálamo ramifica-se no sistema límbico, onde os aspectos emocionais e afetivos são integrados na experiência da percepção da dor (AGUGGIA, 2003).

B. Inflamação e Hiperalgesia inflamatória

Consideramos a inflamação como um padrão de resposta estereotipado do organismo a uma lesão ou processo infeccioso. O médico romano Celsus (século I DC.) descreveu o processo inflamatório contendo quatro sinais cardinais: rubor (vermelhidão), tumor (inchaço), calor (aumento local de temperatura) e dolor (dor). Já em meados do século 19 um quinto sinal cardinal da inflamação foi incorporado nesta descrição por Rudolf Virchow: Functio laesa (perda de função da região ou órgão afetado pelo processo inflamatório). Três dos sinais cardinais da inflamação; vermelhidão, inchaço e aumento de temperatura; derivam do aumento de fluxo sanguíneo e da permeabilidade da vasculatura. Ao passo que o sinal cardinal dor está relacionado a mudanças no sistema sensorial relacionado a esta percepção (Ferreira, 1972; Libby, 2007). Suas principais características são acúmulo de líquidos e proteínas plasmáticas (edema), migração celular e sensibilização ou ativação dos nociceptores. Essa resposta pode ser causada por trauma mecânico, privação de oxigênio ou nutrientes, alterações imunológicas ou

genéticas, agentes químicos, micro-organismos, temperaturas extremas ou radiação (ROTE et al., 1998; SHERWOOD; TOLIVER-KINSKY, 2004).

A dor, qual é tema de interesse neste estudo, é um dos sintomas mais frequentes na prática clínica e um dos problemas de tratamento mais importantes. Em algumas situações, pode diminuir com a simples administração de medicamentos e, em outras, pode ser objeto de difícil terapêutica.

Fenômenos paralelos podem acompanhar a dor como a hiperalgesia, que resulta da sensibilização das fibras neuronais sensoriais responsáveis pela detecção dos estímulos nocivos capazes de ativar o sistema nociceptivo. Essa sensibilização tem como característica a diminuição do limiar de excitabilidade neuronal (RIEDEL; NEECK, 2001), devido à ação de mediadores inflamatórios produzidos no sitio do dano tecidual (HUANG; ZHANG; MCNAUGHTON, 2006; VERRI et al., 2006).

A hiperalgesia inflamatória é um fenômeno comum a todas as dores de origem inflamatória e é induzida pela sensibilização dos nociceptores por mediadores inflamatórios liberados no sítio do dano tecidual (COUTAUX et al., 2005). Esses mediadores inflamatórios, através da ativação de seus respectivos receptores, induzem alterações metabólicas neuronais que resultam na sensibilização dos nociceptores. Portanto, ao contrário da nocicepção, que é considerada um fenômeno puramente iônico, a hiperalgesia é um fenômeno metabólico (CATERINA; JULIUS, 2001; LEWIN; LU; PARK, 2004; RAJA; HAYTHORNTHWAITE, 1999; SNIDER; MCMAHON, 1998).

A sensibilização caracteriza-se pela diminuição do limiar de ativação dos nociceptores, os quais tendem a disparar potenciais de ação mais facilmente, de maneira que estímulos de menor intensidade (antes inofensivos) tornam-se capazes de disparar um potencial de ação (CUNHA et al., 2005). A sensibilização dos nociceptores é caracterizada eletrofisiologicamente não só pela diminuição do limiar de excitabilidade dos nociceptores como também pelo aumento da atividade espontânea do neurônio e pelo aumento na frequência de disparo em resposta a estímulos de baixa intensidade (RIEDEL; NEECK, 2001).

Durante o processo inflamatório ocorre a liberação de eicosanóides, que sensibilizam diretamente os nociceptores. Dentre os eicosanóides, estão as prostaglandinas (PGs), formadas pela ação da enzima ciclo-oxigenases (COX) (FERREIRA; VANE, 1967). Trabalhos demonstram que as PGs, principalmente da série E, são mediadores que causam hiperalgesia inflamatória (FERREIRA, 1973). A participação das PGs na sensibilização dos nociceptores tem sido observada em humanos e animais, desde o século passado, com o uso de técnicas eletrofisiológicas e comportamentais (CHAHL; IGGO, 1977; HANDWERKER; NEHER, 1976; PERL et al., 1976). Além disso, várias evidências sugerem que principalmente as PGs do tipo E2 são responsáveis por induzir a hiperalgesia (FERREIRA, 1972).

A hiperalgesia também pode resultar da ativação de uma via independente de PGE_2 que envolve a liberação de aminas simpatomiméticas. Por exemplo, a administração intraplantar (i.pl.) de agonistas adrenérgicos, como a noradrenalina, a adrenalina, a isoprenalina, a dopamina ou de aminas simpatomiméticas de ação

indireta, como a tiramina, induzem hiperalgesia de intensidade semelhante à induzida pelas PGs, via ativação dos receptores dopaminérgicos D1 e β-adrenérgicos (NAKAMURA; FERREIRA, 1987). Apesar das PGs e aminas simpatomiméticas constituírem os principais mediadores finais da hiperalgesia inflamatória, a liberação desses mediadores é precedida pela liberação de outros como a bradicinina (BK) e as citocinas.

A bradicinina é uma cinina (hormônios teciduais de natureza polipeptídica que afetam a musculatura lisa e possuem propriedades vasoativas) que é liberada a partir de globulinas plasmáticas pela ação da tripsina (STANISAVLJEVIC et al., 2006), participa da hiperalgesia inflamatória via ativação dos receptores da BK (B1 e B2) e da posterior liberação da citocina TNF-α e, resultanto em aumento da permeabilidade plasmática (POOLE et al., 1999). Vários estudos demonstraram que tanto o TNF-α como as citocinas IL-1, IL-6, IL-8 induzem a sensibilização dos nociceptores em vários modelos experimentais (CUNHA et al., 2005; DAVIS; PERKINS, 1994; HRUBEY et al., 1991; PERKINS; KELLY; DAVIS, 1995). O tratamento com guanetidina (droga que induz a depleção de aminas endógenas periféricas), com antagonistas β-adrenérgicos ou com antagonistas de receptores dopaminérgicos é capaz de inibir a hipernocicepção induzida por IL-8 (CUNHA et al., 1991). Os mesmos efeitos foram demonstrados em relação ao CINC-1 (do inglês citokine-induced neutrophil chemoattractant) (LORENZETTI et al., 2002).

Tem sido sugerida a existência de uma cascata de liberação de citocinas na hiperalgesia mecânica (avaliada pelo método de filamentos de von Frey) e térmica (avaliada no método de placa quente) (SAFIEH-GARABEDIAN et al.,

1995; WOOLF et al., 1997). Entretanto, a liberação dos mediadores hiperalgésicos de forma hierárquica é controversa.

Alguns mediadores inflamatórios além de sensibilizar os nociceptores, fazendo com que respondam a estímulos anteriormente não nociceptivos, podem estimular diretamente os nociceptores levando a dor inflamatória espontânea. Recentemente, tem sido descrito que tanto a sensibilização dos nociceptores quanto a nocicepção inflamatória é resultado da ação interdependente ou sinérgica de vários mediadores inflamatórios. Enquanto alguns mediadores precedem a liberação de mediadores finais, que sensibilizam ou estimulam diretamente as fibras nociceptivas primárias, outros atuam diretamente nos nociceptores para aumentar a sua susceptibilidade aos mediadores que os sensibilizam ou estimulam (PARADA; YEH; JOSEPH; et al., 2003; PARADA; YEH; REICHLING; et al., 2003).

A carragenina é um polissacarídeo sulfatado derivado de algas vermelhas (*Rhodophycae*), amplamente utilizada como um agente estabilizante, espessante e emulsificante em muitos alimentos processados na dieta ocidental, como também em vários outros produtos, incluindo cosméticos, creme dental, aromatizante de ambiente e produtos farmacêuticos (BORTHAKUR et al., 2012). Estudos em mamíferos tem demonstrado que exposição prolongada causa inflamação, incluindo desenvolvimento de ulcera, colite e tumores colorretais (TOBACMAN; WALTERS, 2001). A carragenina é utilizada como agente inflamatório por provocar uma reação inflamatória local aguda resultando em dor, edema e hiperalgesia, que são os sintomas mais frequentes das dores

inflamatórias em seres humanos (BORTHAKUR et al., 2012). A administração periférica da carragenina tem sido amplamente utilizada como modelo de estudo da hiperalgesia inflamatória uma vez que ela induz a sensibilização dos nociceptores para estímulos mecânicos (KAYSER; GUILBAUD, 1987; OSBORNE; CODERRE, 1999; VINEGAR et al., 1987; ZHANG et al., 1997), induz a migração de neutrófilos (ALMEIDA et al., 1980; PINHEIRO; CALIXTO, 2002; VINEGAR et al., 1987), e promove a manifestação de outras características da resposta inflamatória.

A carragenina, ao induzir a formação de bradicinina e a liberação de citocinas, sobretudo TNFα, IL-1β, IL-6 e IL-8, induz a produção de prostaglandinas e a liberação de aminas simpatomiméticas que medeiam, em última instância, a hiperalgesia observada nesse modelo (BORTHAKUR et al., 2012; FERREIRA, 1973). A injeção intra-articular ou intraplantar unilateral de carragenina em ratos é um modelo experimental de dor aguda utilizado em vários estudos (MILLAN et al., 1988; MIN et al., 2001; OLIVEIRA et al., 2005; SLUKA et al., 1997; SOLANO; HERRERO, 1999; TONUSSI; FERREIRA, 1992) inclusive nos que avaliam a eficácia de medicamentos anti-inflamatórios, e de medicamentos destinados a aliviar a inflamação associada a dor e edema (BENNETT; XIE, 1988; LAWAND; MCNEARNEY; WESTLUND, 2000; MUIRDEN; PEACE, 1969). A carragenina induz dor e hiperalgesia mediada pela liberação de dois mediadores inflamatórios finais, as prostaglandinas e as aminas simpatomiméticas, que sensibilizam diretamente as fibras aferentes primárias

(GOLD et al., 1996; RUSH; WAXMAN, 2004). A produção desses mediadores finais depende da liberação prévia de uma cascata de citocinas, que envolve inicialmente a formação da bradicinina, que por sua vez, induz à liberação da citocina pró-inflamatória TNFα (FERREIRA, 1973; PERKINS; KELLY; DAVIS, 1995). Esta citocina desencadeia a liberação de duas vias distintas de citocinas, uma mediada pela IL-1β e IL-6 que estimula a síntese da ciclo-oxigenase-2 (COX-2) convertendo o ácido araquidônico em prostaglandinas e outra mediada pela IL-8 (em humanos) ou CINC-1 (em ratos) que estimula a produção de aminas simpatomiméticas (CUNHA et al., 1991; LORENZETTI et al., 2002).

C. TRPA1

O receptor de TRPA1 é o mais recente membro identificado da superfamília de receptores TRP (Transiente Receptor Potencial) originalmente conhecido como ANKTM1 (*ankyrin-like with transmembrane domains protein 1*) identificado primeiramente em fibroblastos de pulmões fetais de humanos (JAQUEMAR; SCHENKER; TRUEB, 1999). Estudos posteriores mostraram que o receptor de TRPA1 também é expresso em neurônios sensoriais dos gânglios da raiz dorsal (DRG), gânglio nodoso (NG), neurônios dos gânglios trigeminais (TG) e queratinócitos (ATOYAN; SHANDER; BOTCHKAREVA, 2009; KWAN et al., 2009; NAGATA et al., 2005; STORY et al., 2003), e em neurônios simpáticos pós-ganglionares, neurônios motores e neurônios do plexo mioentério intestinal (SMITH et al., 2004). O receptor de TRPA1 é um canal de cátions não seletivo (LEVINE; ALESSANDRI-HABER, 2007) que inicialmente foi associado à detecção

de temperaturas frias nocivas (STORY et al., 2003), e só recentemente ganhou destaque como um receptor importante na condução da informação nociceptiva em condições inflamatórias (ANDRADE et al., 2008; BANDELL et al., 2004; DAI et al., 2007; MACPHERSON; DUBIN; et al., 2007; MCNAMARA et al., 2007; PETRUS et al., 2007; WANG et al., 2008). Sua importância na dor inflamatória foi ressaltada quando Story et al. (2003) demonstraram co-expressão entre TRPV1 e TRPA1 em muitos neurônios de pequeno diâmetro que contém substância P e o peptídeo relacionado ao gene da calcitonina (CGRP) sugerindo sua expressão em fibras Aδ e C que são altamente responsivas à estímulos nocivos.

Estruturalmente o receptor de TRPA1 é composto por 6 domínios transmembrana tendo ao menos quatorze repetições *ankyrin* na sua porção N-terminal intracelular, a qual deriva seu nome (STORY et al., 2003). O receptor TRPA-1 pode ser ativado diretamente, por meio de ligações covalentes inespecíficas (HINMAN et al., 2006; MACPHERSON; XIAO; et al., 2007) e as primeiras substâncias químicas identificadas como capazes de ativá-lo foram os compostos irritantes presentes em extratos de alho, o alicin (MACPHERSON et al., 2005), e de mostarda, o alil isotiocianato (JORDT et al., 2004). Recentemente, foi demonstrado que substâncias endógenas também ativam diretamente o TRPA1, como no caso de metabólitos ativos das prostaglandinas E2 e D2 (TAYLOR-CLARK et al., 2009) e do HNE (4-hydroxy-2-nonenal) um aldeído endógeno produzido em altas concentrações através da peroxidação dos fosfolipídios de membrana em resposta a injúria tecidual e inflamação

(MACPHERSON; DUBIN; et al., 2007; TREVISANI et al., 2007). Além da ativação direta por substâncias endógenas e exógenas, tem sido demonstrado que o receptor de TRPA1 funciona como um canal operado por receptores, ou seja, mediadores inflamatórios liberados no sítio da injúria ativam seus respectivos receptores e iniciam vias de sinalização celular que levam a ativação do receptor de TRPA1. Por exemplo, foi demonstrado que a hiperalgesia induzida pela administração de bradicinina na pata depende do receptor de TRPA1 que é sensibilizado em resposta a ativação da proteína quinase A (PKA) e da via da fosfolipase C (PLC) pelo receptor de bradicinina B2 (BANDELL et al., 2004; BAUTISTA et al., 2006; WANG et al., 2008). A PLC e a PKA podem ser ativadas por inúmeros mediadores inflamatórios e estão entre os principais mecanismos responsáveis pela sensibilização dos nociceptores, o que evidencia a importância do receptor de TRPA1 nesse processo de sensibilização.

O papel do receptor de TRPA1 no processo de sensibilização dos nociceptores periféricos tem sido pouco investigado, mas já foi demonstrado que o TRPA1 medeia a hiperalgesia induzida pelo completo adjuvante de Freund (CFA) (DA COSTA et al., 2010; DUNHAM; KELLY; DONALDSON, 2008; EID et al., 2008; OBATA et al., 2005; PETRUS et al., 2007), o que indica que a ativação ou sensibilização do receptor de TRPA1 pelos diferentes mediadores inflamatórios pode contribuir para a diminuição do limiar nociceptivo e consequentemente, para a hiperalgesia inflamatória. O CFA é uma emulsão oleosa que contém micobactérias mortas por calor (“heatkilled”), as quais misturadas em excipiente oleoso persiste por semanas ou mesmo meses no local

injetado, e também um imunomodulador ativo (muramil-dipeptídeo) que se liga a receptores de reconhecimento de padrões associadas à patógenos em células do sistema imune inato. Esta ligação inicia uma cascata de sinais que desencadeiam uma resposta imune inata (GUY, 2007; KENSIL; MO; TRUNEH, 2004). Dados que suportam essa hipótese demonstram que a bradicinina, um mediador liberado no início do processo inflamatório induzido pela carragenina (FERREIRA; LORENZETTI; POOLE, 1993) ativa o TRPA1 através da PLC (BAUTISTA et al., 2006) e que metabólitos das prostaglandinas, que são os mediadores finais responsáveis pela sensibilização induzida pela carragenina (FERREIRA; LORENZETTI; POOLE, 1993), ativam diretamente o receptor de TRPA1 (TAYLOR-CLARK et al., 2009).

Portanto, é possível que o receptor de TRPA1 medeie a hiperalgesia inflamatória mecânica induzida no modelo da carragenina. Considerando que o receptor de TRPA1 é expresso principalmente em neurônios nociceptivos primários, é provável que o TRPA1 neuronal contribua com a hiperalgesia inflamatória. No entanto, ainda não está claro se estes receptores são expressos em células inflamatórias ativadas, embora sejam expressos em queratinócitos. A presença do receptor de TRPA1 presença tanto em fibras nociceptivas quanto em queratinócitos sugere que este canal promove uma comunicação entre os terminais nociceptivos e os queratinócitos, modulando a mecanotransdução (ATOYAN; SHANDER; BOTCHKAREVA, 2009). Nesse contexto, a ativação destes receptores em queratinócitos poderia indiretamente induzir a liberação de mediadores inflamatórios, de forma que o receptor de TRPA1 poderia contribuir

com a hiperalgesia inflamatória também através de uma atuação indireta nos nociceptores, mediada pela liberação de mediadores inflamatórios.

II. PROPOSIÇÃO

O objetivo desse trabalho foi testar a hipótese de que o TRPA-1 medeia a hiperalgesia inflamatória mecânica induzida pela administração de carragenina na pata de ratos e investigar os mecanismos envolvidos.

III. Capítulo

The role of transient receptor potential A 1 (TRPA1) in the development and maintenance of carrageenan-induced hyperalgesia.

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Original Article

Keywords: TRPA1; Carrageenan; nociceptor sensitization, hyperalgesia, inflammation; cytokines; neutrophil migration

Abstract

Transient receptor potential ankyrin 1 (TRPA1) is a nonselective cation channel important in setting nociceptive threshold. It is expressed in nociceptive C-fibers and in non-neuronal cells involved in pro-inflammatory mediators' release. We asked whether TRPA1 contributes to carrageenan-induced hyperalgesia in rats, and if so, whether this contribution is mediated by mechanisms involved in inflammation such as cytokine release and neutrophil migration and/or by a direct sensitization of the primary afferent nociceptors. Pharmacological blockade of local TRPA1 by its selective antagonist HC 030031 prevented and reversed carrageenan -induced hyperalgesia, which was detected either by a mechanical or chemical (low dose of capsaicin) stimulus. However, it did not affect either carrageenan-induced cytokines expression or neutrophil migration. The neuronal TRPA1 gene silencing induced by intrathecal pre-treatment with antisense oligodoexynucleotide completely prevented carrageenan-induced hyperalgesia over 24 hours and significantly reduced TRPA1 expression in the dorsal root ganglia cells (L5-6), which was not affected by carrageenan treatment. We conclude that TRPA1 plays an important role in the development and maintenance of carrageenan-induced inflammatory hyperalgesia by directly contributing to nociceptor excitability.

Introduction

Transient receptor potential ankyrin 1 (TRPA1) is the most recently identified mammalian member of the TRP superfamily. This nonselective cation channel is primarily expressed in a subset of TRPV1- expressing peptidergic nociceptors, where its activation contributes to nociceptor excitability and neurogenic inflammation (Story et al., 2003). However, the recent discovery that TRPA1 is also expressed in non-neuronal cells (Atoyan et al., 2009; Jain et al., 2011; Prasad et al., 2008) has brought new insights into the function of this receptor in inflammatory pain. As a sensor of chemical damage, TRPA1 can be activated by a wide range of mechanisms. It is directly activated by intracellular calcium (Doerner et al., 2007) and numerous endogenous and exogenous reactive compounds via covalent modification (Bautista et al., 2006; Jordt et al., 2004; Trevisani et al., 2007). TRPA1 is also indirectly activated, by inflammatory mediators through signaling pathways that are activated by their action on G-protein-coupled and other membrane receptors (Bautista et al., 2006; Wang et al., 2008). Because of this gating promiscuity, TRPA1 has emerged as a major mediator of inflammatory pain. However, whether TRPA1 contributes to inflammatory pain by mechanisms involved in inflammation, e.g. cytokine release and neutrophil migration is not known.

The injection of carrageenan into the rodent's hind paw is a widely used model for investigating the inflammatory pain response and screening novel drugs for pain treatment and control (Winter et al., 1962). It induces the production of bradykinin, which triggers the pro-inflammatory cytokines release from non-

neuronal cells resulting in neutrophil migration (Cunha et al., 2008; Ferreira et al., 1993). Neutrophils, in turn, release direct-acting hyperalgesic mediators, such as PGE_2, that ultimately sensitize the nociceptors, lowering their firing threshold (Cunha et al., 2008). In this study, we investigated, whether TRPA1 contributes to carrageenan-induced hyperalgesia, and, if so, whether this contribution is mediated by mechanisms involved in inflammation such as cytokine release and neutrophil migration and/or by a direct sensitization of the primary afferent nociceptors.

Material and methods

Subjects

Male Wistar rats (200-300 grams) were used. The experiments were in accordance with IASP guidelines for the study of pain in animals (Zimmermann, 1983). All animal experimental procedures and protocols were approved by the Committee on Animal Research of the State University of Campinas. The animals were housed in plastic cages with soft bedding (five/cage) on a 12-h light/dark cycle (lights on at 6:00 a.m.) in a temperature-controlled (± 23°C) vivarium. Food and water were available *ad libitum.*

Drugs

Carrageenan (50, 100 or 300 µg) and capsaicin (0.5 µg) were obtained from Sigma- Aldrich (St. Louis, MO, USA), the TRPA1 antagonist HC 030031 (300 or 1200 µg) was obtained from Tocris Bioscience (Bristol, UK, USA). Carrageenan was freshly prepared and dissolved in 0.9% NaCl (sterile saline), capsaicin was dissolved in 0.9% NaCl and HC 030031 in dimethyl sulfoxide (DMSO). All drugs were injected in the subcutaneous tissue of the plantar surface of the rat's hind paw.

Behavioral tests

Testing sessions took place during light phase (between 09:00 AM and 5:00 PM) in a quiet room maintained at 23 °C. Before the experiments, each animal was manipulated for 7 days to be habituated to the experimental manipulation. On the

day of the experiment, each animal was individually placed in the test chamber 15 min before the beginning of the tests to minimize stress. Rats did not have access to food or water during the test and each animal was used once.

Mechanical hyperalgesia: The test chamber consists of acrylic cages (12 x 20 x 17 cm high) with a wire grid floor. A tilted mirror below the grid provided a clear view of the animal's hind paw. Before testing, the animals were quiet, without exploratory movements and not resting over the paws. The test consisted of evoking a hind paw flexion reflex with a handheld force transducer (electronic anesthesiometer, IITC Life Science, Woodland Hills, CA, USA) fitted with a 0.5 mm^2 polypropylene tip. The tip is applied to the central area of the plantar hind paw with a gradual increase in pressure. After paw withdrawal, the intensity of the pressure was automatically recorded. The test was performed at each defined time intervals, for example, for figure 1A at 0, 0.5, 1.0, 2.0 3.0, 6.0 and 24 hours after injection, during test intervals animals returned to their house-cages. The baseline paw-withdrawal threshold was defined as the mean of three tests performed before intraplantar injection, being 60 g the upper limit pressure. The nociceptive threshold is defined as the mean of three tests performed, at each time interval after intraplantar injection. Mechanical hyperalgesia was quantified as the change in the mechanical nociceptive threshold, expressed by the delta (Δ) withdrawal threshold (in g) calculated by subtracting the nociceptive threshold, at each indicated times after injection, from the baseline threshold.

Overt Nociception: The test chamber consists of a mirrored-wood chamber (30 x 30 x 30 cm) with a glass at the front side. Following the intraplantar injection,

the behavioral nociceptive response was quantified by counting the number of spontaneous flinches/shakes of the injected hind paw during 5 min.

Measurement of myeloperoxidase activity

Myeloperoxidase is one of the enzymes released from neutrophils and directly associated to tissue injury. Although monocites/macrophages (Klebanoff, 1980) and fibroblasts also contain myeloperoxidase (Nozawa-Inoue et al., 2003), neutrophils show the highest intracellular levels of this enzyme, that represents up to 5% of neutrophil proteins (Klebanoff, 1991).

The myeloperoxidase kinetic-colorimetric assay was used, as previously described (Torres-Chavez et al., 2012). Three hours after injection, the tissue of the plantar surface of the hind paw was dissected into a standard sized sample, weighed, quickly frozen by liquid isopentane in dry ice and stored at -70^{o} C. In the assay procedure, each sample was homogenized in 500 µl of buffer 1 (0.1M NaCl, 0.02 M $NaPO_4$, 1.015 M Na EDTA, pH 4.7) followed by centrifugation at 3000 rpm for 15 min. The pellet was resuspended in 500 µl of buffer 1 and subjected to hypotonic lyses by the addition of 500 µl of 0.2% NaCl followed 30 s later by addition of 500 µl of 1.6% NaCl in 5% glucose. After a further centrifugation, the pellet was resuspended in 0.05 M NaPO4 buffer (pH 5.4) containing 0.5% hexadecyl-trimethylammonium bromide (HTAB). After that, the samples were snap-frozen in liquid nitrogen and thawed, three times, and centrifuged at 10000 rpm for 15 min. Fifty microliters of each sample (supernatant) and of 0.08 M NaPO4 were dropped into wells of a 96-well microplate. 25 µl of 3,3′, 3,3′ -

tetramethylbenzidine was added in each well and the reaction was initiated by the addition of 100 µl of H_2O_2. The reaction was stopped 5 min later by the addition of 50 µl of 4M H_2SO_4. The optical density was read at 450 nm using an Anthos 2020. Results were calculated by comparing the optical density of hind paw tissue supernatant with a standard curve of neutrophil (>95% purity) obtained as previously described (Torres-Chavez et al., 2012). The results were presented as number of neutrophils $x10^6$/mg tissue.

Cytokines measurement

Three hours after injection, the tissue of the plantar surface of the hind paw was dissected into a standard sized sample, weighed, snap frozen on dry ice and store at -70^o C. The time-point for cytokines measurement was defined based on the time course of carrageenan-induced inflammatory hyperalgesia. Cytokines measurement was performed as previously described (Torres-Chavez et al., 2011). Prior to the assay, hind paw tissue was homogenized in phosphate buffered saline (PBS, pH=7.4) containing 0.4 M NaCl, 0.05 % Tween-20, 0.5 % bovine serum albumin, 0.1 mM phenylmethylsulfonyl fluoride, 0.1M benzethonium chloride, 10 mM EDTA and 20 Kl/mL aprotinin. The homogenenates were centrifuged at 12000 RPM for 15 min at 4^{o}C. Two-site ELISA was used to measure the cytokines TNF-α, IL1-β and IL-6 using an immunoassay kit (DuoSet, R&D) as described by the manufacturer. The results are expressed as picograms (pg) of each cytokine per gram (g) of hind paw tissue.

Antisense oligodeoxynucleotides (ODNs)

The functional blockade of TRPA1 receptor expression on peripheral sensory neurons was performed by the intrathecal injection of antisense ODN. The following antisense ODN sequence was used: 5´- TATCGCTCCACATTGCTAC -3` The mismatch-ODN sequence, 5`- ATTCGCCTCACATTGTCAC -3`, corresponded to the antisense sequence except that six bases were changed. A search on the NCBI database to *Rattus norvegicus* identified no other sequences homologous to that used in this experiment. The antisense ODNs were synthesized by Erviegas (São Paulo, SP, Brazil), lyophilized and reconstituted in 0.9% NaCl.

Antisense ODN was intrathecally injected as previously described (Fischer et al., 2005; Papir-Kricheli et al., 1987). Briefly, for each injection, rats were anesthetized with 1/3 O_2 to 2/3 N_2O and halothane at 5% and 1.5%, respectively (OLIVEIRA et al., 2005). A 26-gauge needle was inserted in the subarachnoid space on the midline between L5 and L6 vertebrae. The animals regained consciousness approximately 1 min after discontinuing the anesthetic. A dose of 5 nmol of TRPA1 antisense- or mismatch-ODN (Andrade et al., 2008) was intrathecally administered in a volume of 10 µl (at 1 µl/s) once daily for 4 days. Experiments were initiated 1 hour after the last injection.

Western-blot analysis of TRPA1 expression

The immunoblot study was conducted as previously described (Romero-Calvo et al., 2010) to assess the efficacy of antisense-ODN treatment and to quantify TRPA1 expression after carrageenan injection. Previously stored L5-L6

DRG samples were homogenized with an ultrasonic homogenizer (Sonic Corporation, USA), in a buffer containing 1% Triton X-100, 50 mM Phosphate Buffer, pH 7.4, 8M Urea, 2M thiourea, 1mM EDTA, 1% complete protease inhibitor cocktail (Sigma, USA - P8340), at 4ºC. After 20 minutes incubation at 4ºC, the samples were centrifuged at 12,000g for 15 minutes at 4ºC, and the resulting supernatant was transferred to a new tube. Protein concentration was determined by the Bradford method, and 70 ug of total proteins from each sample were separated by SDS-PAGE and transferred to nitrocellulose membranes according to standard techniques. Membranes were stained with Ponceau S, and digital images were acquired for the control of protein loading by densitometric analysis (Romero-Calvo et al., 2010). Membranes were blocked with 5% non-fat dry milk solution in PBS containing 0,1% tween-20, and incubated overnight with anti-TRPA1 (1:1000), followed by HRP conjugated secondary antibody (Zymed, 1:10000). Reactive bands were detected with the Super Signal West Pico chemiluminescent kit. Results are expressed as the ratio between TRPA1 and the corresponding Ponceau S optical densities.

Statistical analysis

A two-way repeated measures ANOVA with one between-subjects factor (i.e., treatment) and one within-subjects factor (i.e., time) was used to determine whether there were significant differences among the groups in figures 1 A, 1 B and 3 A. To determine if there were significant differences between treatment groups in figures 1 C and 2 one-way ANOVA was performed. If there was a significant

between-subjects main effect of treatment group following ANOVA, post hoc contrasts using the Tukey test were performed to determine the basis of the significant difference. To determine if there were significant differences between treatment groups in figures 3 B and C a *t*-test was performed. The level of significance was set at $p < 0.05$. Data are expressed in figures by intensity of mechanical hyperalgesia (figures 1 A, 1 B and 3 A), by number of paw flinches (figure 1 C), by amount of cytokines (pg) per gram of tissue (figure 2 A, 2 B and 2 C), by the number of neutrophils per gram of tissue (figure 2 D) or by TRPA1 expression (relative optical density, figures 3 B and C) and are presented as means ± S.E.M.

Results

Carrageenan-induced inflammatory hyperalgesia

The subcutaneous administration of carrageenan into the plantar surface of the rat hind paw significantly reduced the mechanical paw withdraw threshold, referred as hyperalgesia, in a dose and time-dependent manner. The hyperalgesic effect peaked at 3 hours and was still present at 24 hours after the carrageenan administration. The 100 µg dose of carrageenan induced a mechanical hyperalgesia similar in magnitude to that induced by 300 µg and significantly higher than that induced by 50 µg (Figure 1A). Therefore, the 100 µg dose was used in further experiments. Two-way repeated-measures ANOVA showed a significant main effect of treatment ($F = 567.60$; $p < 0.001$) and a significant treatment x time interaction ($F = 38.68$; $p < 0.001$).

Effect of pharmacological blockade of TRPA1 on carrageenan-induced inflammatory hyperalgesia

To evaluate the contribution of TRPA1 to carrageenan-induced mechanical inflammatory hyperalgesia, TRPA1 was pharmacologically blocked by its selective antagonist HC 030031. HC 030031 was either co-administrated with carrageenan or administrated 2 hours and 55 minutes after the carrageenan administration, that is, 5 minutes before the time at which carrageenan-induced hyperalgesia is maximal. The co-administration of HC 030031 (at 1200 µg, but not at 300 µg) significantly reduced carrageenan-induced mechanical hyperalgesia at 3 but not 6 hours after its administration (Figure 1 B). The post-administration of HC 030031 (1200 µg) significantly reduced carrageenan-induced mechanical hyperalgesia at 3

and 6 hours after the carrageenan administration (Figure 1 B). Two-way repeated-measures ANOVA showed a significant main effect of treatment ($F = 600.37$; $p < 0.001$) and a significant treatment x time interaction ($F = 199.67$; $p < 0.001$).

The administration of HC 030031 (1200 µg) into the contralateral hind paw had no effect, indicating that the anti-hyperalgesic effect of HC 030031 is mediated by the blockade of local TRPA1 (carrageenan in the ipsilateral plus HC 030031, 32.22 g $\pm$ 0.75, or its vehicle, 32.26 g $\pm$ 0.87, in the contralateral hind paw, t test, $p = 0.97$). HC 030031 did not affect the mechanical threshold evaluated three hours after the intraplantar saline (0.9% NaCl) administration (0.9% NaCl plus HC 030031, 0.71 g $\pm$ 0.11, or its vehicle, 0.40 g $\pm$ 0.24, data not shown, t test, $p = 0.30$).

To further demonstrate the involvement of TRPA1 in carrageenan-induced inflammatory hyperalgesia, we used a chemical rather than a mechanical stimulus to detect carrageenan-induced inflammatory hyperalgesia. The chemical stimulus, a low dose of capsaicin (0.5 µg) that induces minimal nociceptive behavior, was applied 3 hours after the intraplantar administration of carrageenan at the same local. Under this condition, capsaicin-induced behavioral nociceptive response was significantly increased (Figure 1 C) and used as a quantitative measurement of carrageenan-induced hyperalgesia. The co-administration of the TRPA1 receptor antagonist HC030031 (1200 µg) with carrageenan blocked carrageenan-induced increase in capsaicin-induced nociceptive behavior, without affecting capsaicin-induced nociceptive behavior by itself (Figure 1 C, one way ANOVA and

Tukey test, p = <0.001). These findings corroborate the involvement of TRPA1 in carrageenan-induced inflammatory hyperalgesia.

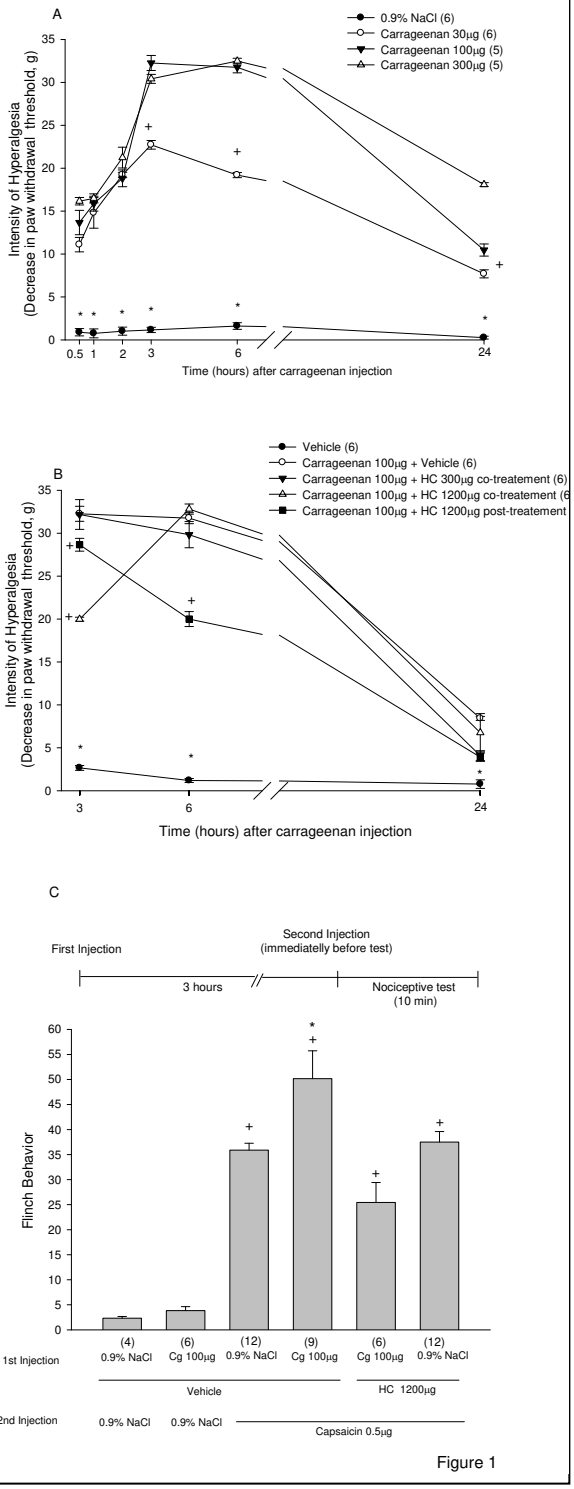

Figure 1

Figure 1- Involvement of TRPA1 on carrageenan-induced inflammatory hyperalgesia.

A- Carrageenan induced a dose and time-dependent hyperalgesia. The symbols "*" and "+" indicate a response significantly lower than that induced by the other groups and by 100 or 300 µg of carrageenan, respectively. There is a statistically significant interaction between treatment and time (F = 38.68; p < 0.001), two way repeated measures ANOVA and Tukey test.

B- The pharmacological blockade of TRPA1 induced by the administration of its selective antagonist, HC 030031, with carrageenan (co-treatment) or two hours and fifty five minutes after carrageenan (post- treatment) significantly decreased mechanical inflammatory hyperalgesia, as indicated by the symbol "+". The symbols "*" indicate a response significantly lower than that of the other groups. There is a statistically significant interaction between treatment and time (F = 116.89; p < 0.001), two way repeated measures ANOVA and Tukey test.

C- The nociception induced by a low dose of capsaicin (third bar) was significantly increased by pre-treatment (three hours before) with carrageenan (fourth bar), as indicated by the symbol "*". This increase was blocked by the selective TRPA1 antagonist HC030031 (fifth bar), which did not affect capsaicin-induced nociceptive behavior by itself (sixth bar). The symbols "+" indicate a response significantly greater (Tukey test, p < 0.05) than that of control groups (first and second bars).

Abreviations:
Vehicle = 0.9% NaCl + DMSO (dimetilsulfoxide)
HC = HC030031, selective TRPA1 antagonist
Cg = carrageenan

Effect of pharmacological blockade of TRPA1 on carrageenan-induced cytokines expression and neutrophil migration

The co-administration of the selective TRPA1 antagonist HC 030031 (1200 µg) did not affect carrageenan-induced either the expression of the cytokines TNF-alpha (Figure 2 A, Tukey test, $p > 0.05$), IL- 1 beta (Figure 2 B, Tukey test, $p > 0.05$) and IL-6 (Figure 2 C, Tukey test, $p > 0.05$) or neutrophil migration (Figure 2 D, Tukey test, $p > 0.05$).

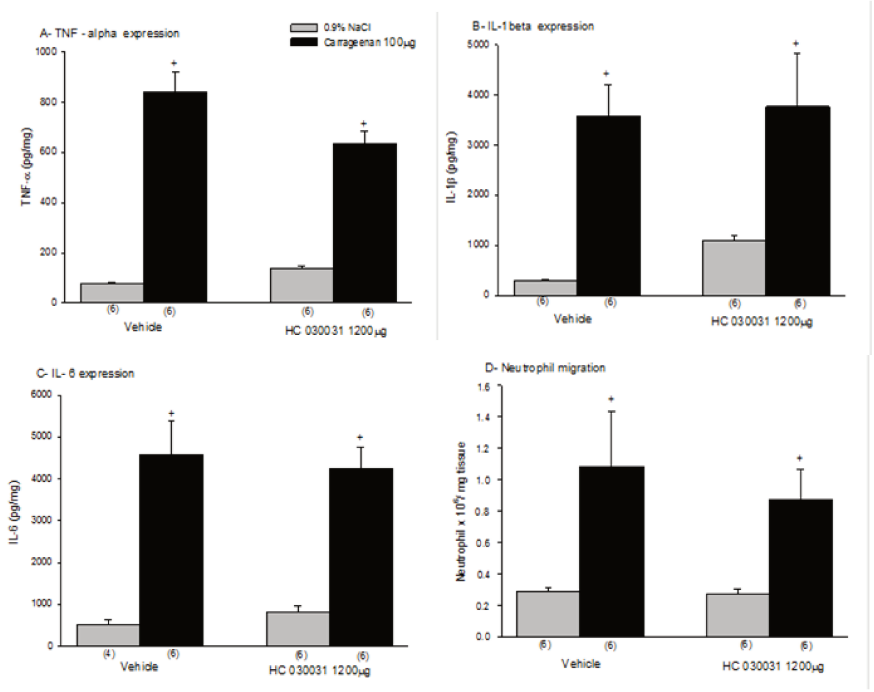

Figure 2- Effect of pharmacological blockade of TRPA1 on carrageenan-induced cytokines expression and neutrophil migration

The pharmacological blockade of TRPA1 by the co-administration of its selective antagonist HC 030031 with carrageenan did not affect carrageenan-induced TNF-alfa (A), IL-1beta (B), IL-6 (C) expression and neutrophil migration (D) (One Waay Anova and Tukey test, $p > 0.05$). The symbols "+" indicate a response significantly greater than that induced by 0.9% NaCl (t test, $p < 0.05$).

Contribution of neuronal TRPA1 to carrageenan-induced hyperalgesia

TRPA1 gene silencing by pre-treatment with antisense-ODN but not with mismatch-ODN (daily intrathecal injection, between L5 and 6, of 5 nmol for 4 days) against TRPA1 prevented carrageenan-induced mechanical inflammatory hyperalgesia (Figure 3 A). Two-way repeated-measures ANOVA showed a significant main effect of treatment ($F = 769.33$; $p < 0.001$) and a significant treatment x time interaction ($F = 511.82$; $p < 0.001$). TRPA1 gene silencing by pre-treatment with antisense-ODN did not affect (0.26 g $\pm$ 0.06) the mechanical paw withdraw threshold (0.40 g $\pm$ 0.25) evaluated three hours after the subcutaneous saline (0.9% NaCl) injection in the hind paw (t test, $p = 0.58$, data not shown).

Treatment with antisense-ODN significantly decreased TRPA1 expression in DRG of L5 and L6 (Figure 3 B, t test $p <0,001$), which provide the neural supply to the plantar surface of the hind paw. The subcutaneous administration of carrageenan into the hind paw did not significantly increase TRPA1 expression in the DRG (L5-6) (Figure 3 C, t test, $p > 0.05$).

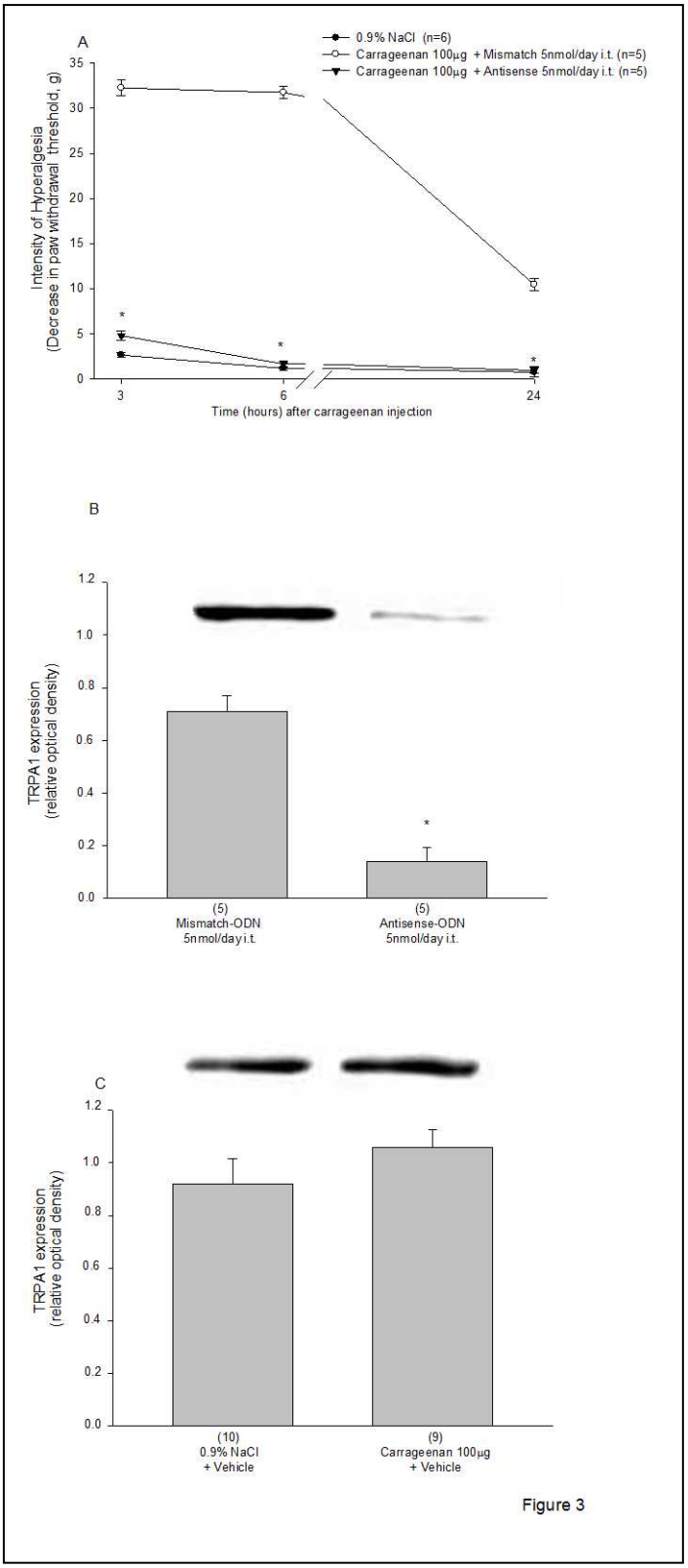

Figure 3

Figure 3- Direct contribution of TRPA1 to carrageenan-induced inflammatory hyperalgesia

A- TRPA1 gene silencing by antisense-ODN (5 nmol daily, intrathecal injection, for four days) prevented carrageenan-induced mechanical inflammatory hyperalgesia, as indicated by the symbols "*". There is a statistically significant interaction between treatment and time ($F = 511,82$; $p < 0.001$), two way repeated measures ANOVA and Tukey test.

B- Treatment with antisense-ODN significantly decreased TRPA1 expression in the dorsal root ganglia (L5 and L6) cells, as indicated by the symbol "*" (t test, $p = <0.001$).

C- Intraplantar injection of carrageenan did not significantly increase TRPA1 expression in the dorsal root ganglia (L5 and L6), t test, $p > 0.05$

Abreviations:

i.t. = intrathecal

Vehicle = 0.9% NaCl + DMSO (dimetilsulfoxide)

HC = HC030031 (selective TRPA1 antagonist)

Discussion

In this study, we showed that neuronal TRPA1 plays an important role in the development and maintenance of carrageenan-induced inflammatory hyperalgesia by directly contributing to nociceptor sensitization.

The selective TRPA1 antagonist HC 030031 prevented and reversed carrageenan -induced mechanical hyperalgesia indicating that TRPA1 is necessary for the development and maintenance of the inflammatory hyperalgesia. The anti-hyperalgesic effect of HC 030031 lasted up to three hours, which is consistent with the previously reported duration of its effect (da Costa et al., 2010). When the effect of HC 030031 disappeared, the hyperalgesic response returned, suggesting that a sustained TRPA1 activation is critical for the maintenance of nociceptor sensitization induced by the inflammatory response. This suggestion is further supported by previous *in vivo* studies showing that TRPA1 mediates long-lasting hyperalgesic responses in different models (da Costa et al., 2010; Dunham et al., 2008; Eid et al., 2008; Obata et al., 2005; Petrus et al., 2007) and by *in vitro* studies showing sustained TRPA1 activation using the perforated patch clamp technique (Koivisto et al., 2012) and intracellular calcium images (Koivisto et al., 2012).

The involvement of TRPA1 in the mechanical transduction in dorsal root ganglia neurons is unclear (Bhattacharya et al., 2008; Kerstein et al., 2009; Rugiero and Wood, 2009). However, here we demonstrated that the pharmacological blockade of TRPA1 or its gene silencing did not affect the mechanical paw withdraw threshold, arguing against a role for this receptor in

nociceptor mechanical transduction. Furthermore, the blockade of TRPA1 significantly reduced carrageenan-induced inflammatory hyperalgesia detected also by a chemical stimulus (low dose of capsaicin), further demonstrating the involvement of TRPA1 in carrageenan-induced inflammatory hyperalgesia.

The carrageenan administration is a classical animal model used to screen drugs for inflammatory pain treatment and control. It induces a widespread inflammatory reaction including edema, cytokines release and neutrophil migration (Ferreira et al., 1993; Winter et al., 1962). We showed that the selective TRPA1 antagonist HC 030031 at a dose that significantly reduced the inflammatory hyperalgesia did not affect cytokines expression and neutrophil migration induced by carrageenan. Therefore, TRPA1 contributes to edema formation (Moilanen et al., 2012) but not to cytokines release or neutrophil migration induced by carrageenan. Taken together, these findings suggest that the role of TRPA1 in carrageenan-induced inflammation may be restricted to edema formation, which is the major consequence of neurogenic inflammation. Although the activation of TRPA1 in nociceptive neurons may contribute to carrageenan-induced cytokines expression and neutrophil migration through neuropeptides release, this contribution is expected to be weak. This is because the major inflammatory function of endogenous neuropeptides is to regulate vascular permeability although the exogenous administration of neuropeptides can induce cytokines expression and neutrophil migration (Ansel et al., 1993; Nakagawa et al., 1995). Despite of its well known role in neurogenic inflammatory mechanisms, the expression of TRPA1 in non-neuronal cells and the more recent evidences that its

activation in these cells leads to the release of cytokines (Atoyan et al., 2009) and the formation of prostaglandins and other inflammatory mediators (Jain et al., 2011) culminate with the idea that TRPA1 may regulate multiple inflammatory mechanisms (Fernandes et al., 2012). However, our data indicate that the important role played by TRPA1 in carrageenan-induced inflammatory hyperalgesia does not depend on cytokines release and neutrophil migration.

The ability of neuronal TRPA1 gene silencing in completely preventing carrageenan-induced hyperalgesia, indicates that the important role of TRPA1 in carrageenan-induced inflammatory hyperalgesia is mediated by a direct contribution to nociceptor excitability, via neuronal TRPA1 activation. The neuronal TRPA1 gene silencing was induced by intrathecal pre-treatment with antisense-ODN against TRPA1 which strongly reduced TRPA1 expression in the dorsal root ganglia cells (L5-6). While TRPA1 located on the peripheral endings of primary afferent nociceptors contributes to inflammatory sensitization (da Costa et al., 2010; Dunham et al., 2008; Eid et al., 2008; Obata et al., 2005; Petrus et al., 2007), those located on central endings facilitate the transmission of nociceptive information to the second order neuron in the spinal dorsal horn (da Costa et al., 2010; Kosugi et al., 2007; Wei et al., 2011). Therefore, the ability of the antisense-ODN in completely preventing carrageenan-induced hyperalgesia probably results from the blockade of central, in addition to peripheral TRPA1-mediated mechanisms.

The mechanisms underlying the sustained TRPA1 activation in response to carrageenan are presently unknown, but may involve bradykinin release which

indirectly gate TRPA1. In addition, carrageenan might induce endogenous TRPA1 agonists release, because many of the oxidants produced during inflammation, such as nitro-oleic acid (Taylor-Clark et al., 2009), 4-hydroxynonenal (Trevisani et al., 2007) or hydrogen peroxide (Andersson et al., 2008) directly gate TRPA1. Therefore, many endogenously produced substances during the inflammatory response triggered by carrageenan may induce TRPA1 activation. Prostaglandin E_2 and sympathetic amines are the most important primary mediators ultimately responsible for inflammatory sensitization (Khasar et al., 1999; Nakamura and Ferreira, 1987). They increase neuronal membrane excitability by activating signaling pathways (cyclic adenosine monophosphate/ protein kinase A (PKA) and phospholipase C (PLC)) that also activate TRPA1 (Wang et al., 2008). Therefore, further studies are warranted to evaluate whether these mediators contributes to TRPA1 activation in the carrageenan model. However, unpublished observations from our labs suggest that Prostaglandin E_2 activates TRPA1 through a PKA dependent mechanism.

In contrast to nerve injury (Frederick et al., 2007; Obata et al., 2005) and the Freund's complete adjuvant (CFA)-induced inflammation (da Costa et al., 2010; Dunham et al., 2008; Obata et al., 2005), carrageenan-induced inflammation did not increase TRPA1 expression in dorsal root ganglia cells of L5 and L6. However, nerve injury and CFA induce long-lasting biochemical changes that strongly modulate gene expression in peripheral nerves, while carrageenan induces a widespread but shorter-lasting inflammatory process, which is maximal at three and almost resolved at twenty four hours. However, it is possible that

carrageenan increases the expression of functional TRPA1 in the cell membrane rather than TRPA1 gene expression. Consistent to this idea, it was demonstrated that TRPA1 membrane insertion is mediated by PKA and PLC (Schmidt et al., 2009), both of which are involved in carrageenan-induced hyperalgesia.

In summary, we showed that TRPA1 plays an important role in the development and maintenance of carrageenan-induced inflammatory hyperalgesia, its neuronal activation directly contributes to nociceptor sensitization. Therefore, a sustained TRPA1 activation seems to be critical for the maintenance of nociceptor sensitization during carrageenan-induced inflammatory hyperalgesia. Based on these findings, we suggest that TRPA1 blockade is a promising strategy for the development of future drugs to pain treatment and control.

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IV. Discussão

Os resultados deste estudo demonstram que o TRPA1 neuronal tem um papel importante no desenvolvimento e manutenção da hiperalgesia inflamatória induzida pela carragenina por contribuir diretamente para a sensibilização do nociceptor.

O antagonista seletivo de TRPA1, HC 030031, preveniu e reverteu a hiperalgesia mecânica induzida pela carragenina indicando que o TRPA1 é necessário para o desenvolvimento e manutenção da hiperalgesia inflamatória. O efeito anti-hiperalgésico do HC 030031 durou cerca de 3 horas, o qual é consistente com resultados prévios da duração do seu efeito (DA COSTA et al., 2010). Quando o efeito do HC 030031 desapareceu, a resposta hiperalgesia retornou, sugerindo que a sustentação da ativação do TRPA1 é crítica para a manutenção da sensibilização induzida pela resposta inflamatória. Isto está de acordo com estudos prévios *in vivo* que demonstram que o TRPA1 medeia a resposta hiperalgésica a longo prazo em diferentes modelos (DA COSTA et al., 2010; DUNHAM; KELLY; DONALDSON, 2008; EID et al., 2008; OBATA et al., 2005; PETRUS et al., 2007) e com estudos *in vitro* que demonstram uma ativação sustentada do TRPA1 através da utilização da técnica de *patch clamp* e imagem de cálcio intracelular (KOIVISTO et al., 2012).

O papel do TRPA1 na transdução mecânica nos neurônios da raiz dorsal (DRG) não está bem elucidado (BHATTACHARYA et al., 2008; KERSTEIN et al., 2009; RUGIERO; WOOD, 2009). No entanto, este trabalho demonstrou que o bloqueio farmacológico do TRPA1 ou o bloqueio da expressão do seu gene não

afeta o limiar mecânico de retirada da pata, argumentando contra o papel desse receptor na transdução nociceptiva mecânica. Além disso, o bloqueio do TRPA1 reduziu significativamente a hiperalgesia inflamatória induzida pela carragenina detectada também por estimulo químico (uma dose baixa de capsaicina), demonstrando mais uma vez o envolvimento do TRPA1 na hiperalgesia inflamatória induzida pela carragenina.

A administração de carragenina é um modelo animal clássico usado para testar drogas para tratamento e controle da dor inflamatória. Isso induz uma reação inflamatória difusa que inclui edema, liberação de citocinas e migração de neutrófilos (FERREIRA; LORENZETTI; POOLE, 1993; WINTER; RISLEY; NUSS, 1962). Os resultados deste estudo demonstram que o antagonista seletivo de TRPA1, HC 030031, reduziu a hiperalgesia inflamatória sem afetar a expressão de citocinas e a migração de neutrófilos induzida pela carregenina. Portanto, o TRPA1 contribui para a formação de edema (MOILANEN et al., 2012), mas não para a liberação de citocinas ou migração de neutrófilos induzida pela carragenina.Esses achados sugerem que o papel do receptor de TRPA1 na inflamação induzida pela carragenina pode ser restrito a formação de edema, que é a maior consequência da inflamação neurogênica. Embora a ativação de TRPA1 nos neurônios nociceptivos pode contribuir para a expressão de citocinas induzidas pela carragenina e para a migração de neutrófilos através da liberação de neuropeptídios, espera-se que esta contribuição seja fraca. Isto porque a principal função dos neuropeptídios endógenos é regular a permeabilidade vascular, embora a administração de neuropeptídios possa induzir a expressão de

citocinas e a migração de neutrófilos (ANSEL et al., 1993; NAKAGAWA; SANO; IWAMOTO, 1995). Independente de seu papel bem conhecido no mecanismo da inflamação neurogênica, a expressão do receptor de TRPA1 em células não neuronais, e as evidências mais recentes de que sua ativação nestas células induz liberação de citocinas (ATOYAN; SHANDER; BOTCHKAREVA, 2009) e a formação de prostaglandinas e outros mediadores inflamatórios (JAIN et al., 2011), culminam com a ideia de que o receptor de TRPA1 pode regular múltiplos mecanismos inflamatórios (FERNANDES; FERNANDES; KEEBLE, 2012). No entanto, nossos dados indicam que o importante papel exercido pelo receptor de TRPA1 na hiperalgesia inflamatória induzida pela carragenina não depende da liberação de citocinas e da migração de neutrófilos.

O silenciamento do gene do receptor de TRPA1 neuronal preveniu a hiperalgesia induzida pela carragenina, indicando que o importante papel do TRPA1 na hiperalgesia inflamatória induzida pela carragenina é mediado por efeito direto sobre a excitabilidade dos neurônios nociceptivos, via ativação de receptor de TRPA1 neuronal. O silenciamento do gene do receptor de TRPA1 foi induzido por pré- tratamento com ODN antisense para receptor de TRPA1 o qual reduziu fortemente a expressão de receptor de TRPA1 nas células dos gânglios da raiz dorsal (L5-6). Enquanto o receptor de TRPA1 localizado nos terminais periféricos dos nociceptores aferentes primários contribuem para a sensibilização inflamatória (DA COSTA et al., 2010; DUNHAM; KELLY; DONALDSON, 2008; EID et al., 2008; OBATA et al., 2005; PETRUS et al., 2007), os que se localizam nos terminais centrais facilitam a transmissão da informação nociceptiva para

neurônios de segunda ordem no corno dorsal da medula espinhal (DA COSTA et al., 2010; KOSUGI et al., 2007; WEI et al., 2011). Por isso, a capacidade do ODN antisense em prevenir completamente a hiperalgesia induzida pela carragenina provavelmente resulta do bloqueio de receptores de TRPA1 centrais e periféricos. Os mecanismos envolvidos na ativação sustentada de receptor de TRPA1 em resposta a carragenina não são conhecidos, mas podem envolver a liberação de bradicinina que indiretamente age no receptor de TRPA1. Além disso, a carragenina poderia induzir a liberação endógena de agonistas de receptor de TRPA1, uma vez que muitos dos oxidantes produzidos durante a inflamação, como o ácido nitro-oleico (TAYLOR-CLARK et al., 2009), 4-hydroxynonenal (TREVISANI et al., 2007) ou peróxido de hidrogênio (ANDERSSON et al., 2008) atuam diretamente sobre o receptor de TRPA1. Portanto, muitas substâncias produzidas endogenamente durante a resposta inflamatória induzida pela carragenina podem induzir a ativação de receptor de TRPA1. A Prostaglandina E2 (PGE2) e as aminas simpatomiméticas são os mais importantes mediadores responsáveis pela sensibilização inflamatória (KHASAR; MCCARTER; LEVINE, 1999; NAKAMURA; FERREIRA, 1987). Eles aumentam a excitabilidade da membrana neuronal por ativação de vias de segundos mensageiros (monofosfato de adenosina cíclico (cAMP, proteino kinase A (PKA) e fosfolipase C (PLC)) que também ativam o receptor de TRPA1 (WANG et al., 2008). Por isso, mais estudos são necessários para elucidar se estes mediadores contribuem para a ativação de receptores de TRPA1 no modelo da carragenina. No entanto,

observações não publicadas do nosso laboratório sugerem que a PGE_2 ativa o receptor de TRPA1 através de mecanismos PKA dependentes.

Em contraste com a lesão do nervo (FREDERICK et al., 2007; OBATA et al., 2005) e a inflamação induzida pelo composto adjuvante de Freund (CFA) (DA COSTA et al., 2010; DUNHAM; KELLY; DONALDSON, 2008; OBATA et al., 2005), a inflamação induzida pela carragenina não aumentou a expressão de receptores de TRPA1 nas células dos gânglios da raiz dorsal de L5 e L6. No entanto, a lesão do nervo e mudanças bioquímicas de longo prazo induzidas por CFA modulam fortemente a expressão genética nos nervos periféricos, enquanto a carragenina induz um processo inflamatório mais generalizado, porém, de duração mais curta, tendo seu pico máximo na terceira hora e desaparecendo após aproximadamente 24 horas. No entanto, é possível que a carragenina aumente a disponibilidade do TRPA1 funcional na membrana celular ao invés da expressão do gene do TRPA1. De acordo com essa ideia, foi demonstrado que a inserção de TRPA1 na membrana é mediada por PKA e PLC (SCHMIDT et al., 2009), os quais por sua vez estão envolvidos na hiperalgesia induzida pela carragenina. Em resumo, este trabalho demonstrou que o receptor de TRPA1 exerce um importante papel no desenvolvimento e manutenção da hiperalgesia inflamatória induzida pela carragenina, e que sua ativação neuronal contribui diretamente para a sensibilização do nociceptor. Por isso, uma ativação sustentada do receptor de TRPA1 parece ser necessária para a manutenção da sensibilização do nociceptor durante a hiperalgesia inflamatória induzida pela carragenina. Baseado nesses achados, pode-se sugerir que o bloqueio de

receptores de TRPA1 é uma estratégia promissora para o desenvolvimento de futuras drogas para o controle e tratamento da dor

V. REFERÊNCIAS

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ANEXOS

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The role of transient receptor potential A 1 (TRPA1) in the development and maintenance of carrageenan-induced hyperalgesia

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ABSTRACT

Transient receptor potential ankyrin 1 (TRPA1) is a nonselective cation channel important in setting nociceptive threshold. It is expressed in nociceptive C-fibers and in non-neuronal cells involved in pro-inflammatory mediators' release. We asked whether TRPA1 contributes to carrageenan-induced hyperalgesia in rats, and if so, whether this contribution is mediated by mechanisms involved in inflammation such as cytokine release and neutrophil migration and/or by a direct sensitization of the primary afferent nociceptors. Pharmacological blockade of local TRPA1 by its selective antagonist HC 030031 prevented and reversed carrageenan-induced hyperalgesia, which was detected either by a mechanical or chemical (low dose of capsaicin) stimulus. However, it did not affect either carrageenan-induced cytokines expression or neutrophil migration. The neuronal TRPA1 gene silencing induced by intrathecal pre-treatment with antisense oligodoexynucleotide completely prevented carrageenan-induced hyperalgesia over 24 h and significantly reduced TRPA1 expression in the dorsal root ganglia cells (L5-6), which was not affected by carrageenan treatment. We conclude that TRPA1 plays an important role in the development and maintenance of carrageenan-induced inflammatory hyperalgesia by directly contributing to nociceptor excitability.

1. Introduction

Transient receptor potential ankyrin 1 (TRPA1) is the most recently identified mammalian member of the TRP superfamily. This nonselective cation channel is primarily expressed in a subset of TRPV1-expressing peptidergic nociceptors, where its activation contributes to nociceptor excitability and neurogenic inflammation (Story et al., 2003). However, the recent discovery that TRPA1 is also expressed in non-neuronal cells (Atoyan et al., 2009; Jain et al., 2011; Prasad et al., 2008) has brought new insights into the function of this receptor in inflammatory pain. As a sensor of chemical damage, TRPA1 can be activated by a *wide range of* mechanisms. It is directly activated by intracellular calcium (Doerner et al., 2007) and numerous endogenous and exogenous reactive compounds via covalent modification (Bautista et al., 2006; Jordt et al., 2004; Trevisani et al., 2007; Leamy et al., 2011). TRPA1 is also indirectly activated, by inflammatory mediators through signaling pathways that are activated by their action on G-protein-coupled and other membrane receptors (Bautista et al., 2006; Wang et al., 2008). Because of this gating promiscuity, TRPA1 has emerged as a major mediator of inflammatory pain. However, whether TRPA1 contributes to inflammatory pain by mechanisms involved in inflammation, e.g. cytokine release and neutrophil migration is not known.

The injection of carrageenan into the rodent's hind paw is a widely used model for investigating the inflammatory pain response and screening novel drugs for pain treatment and control (Winter et al., 1962). It induces the production of bradykinin, which triggers the pro-inflammatory cytokines release from non-neuronal cells resulting in neutrophil migration (Cunha et al., 2008; Ferreira et al., 1993). Neutrophils, in turn, release direct-acting hyperalgesic mediators, such as PGE_2, that ultimately sensitize the nociceptors, lowering their firing threshold (Cunha et al., 2008). In this study, we investigated, whether TRPA1 contributes to carrageenan-induced hyperalgesia, and, if so, whether this contribution is mediated by mechanisms involved in inflammation such as cytokine release and neutrophil migration and/or by a direct sensitization of the primary afferent nociceptors.

2. Material and methods

2.1. Subjects

Male Wistar rats (200–300 g) were used. The experiments were in accordance with IASP guidelines for the study of pain in animals (Zimmermann, 1983). All

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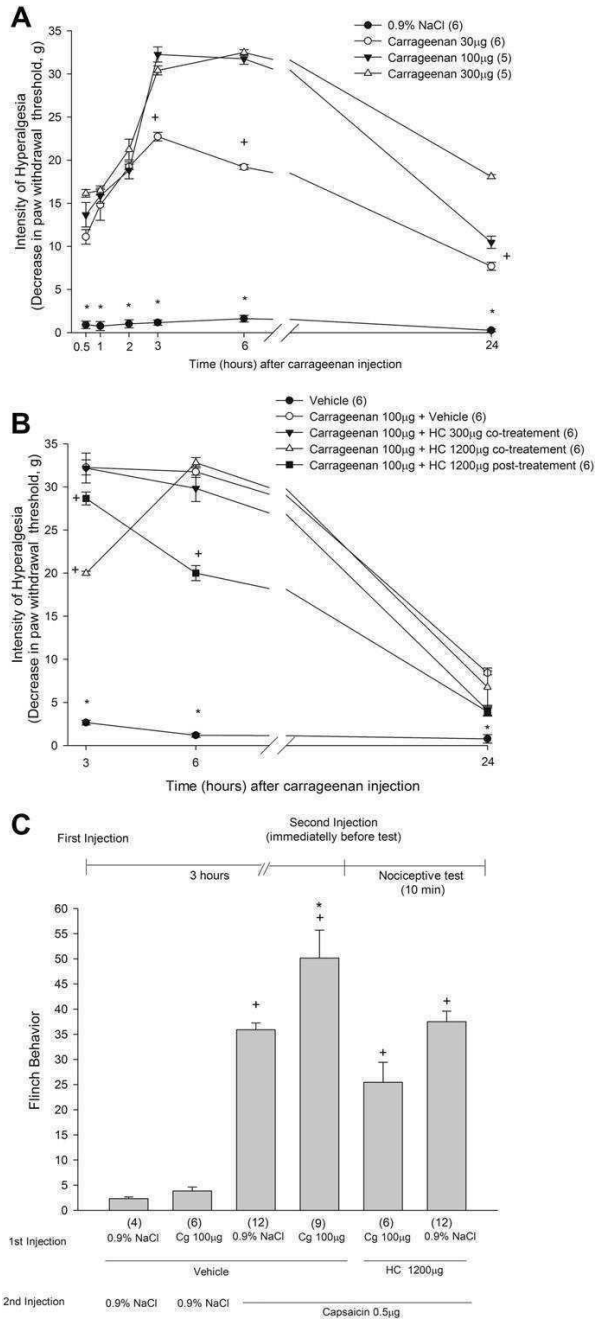

Fig. 1. Involvement of TRPA1 on carrageenan-induced inflammatory hyperalgesia. A – Carrageenan induced a dose and time-dependent hyperalgesia. The symbols "*" and "+" indicate a response significantly lower than that induced by the other groups and by 100 or 300 μg of carrageenan, respectively. There is a statistically significant interaction between treatment and time ($F = 38.68$; $p < 0.001$), two way repeated measures ANOVA and Tukey test. B – The pharmacological blockade of TRPA1 induced by the administration of its selective antagonist, HC 030031, with carrageenan (co-treatment) or two hours and fifty five minutes after carrageenan (post-treatment) significantly decreased mechanical inflammatory hyperalgesia, as indicated by the symbol "+". The symbols "*" indicate a response significantly lower than that of the other groups. There is a statistically significant interaction between treatment and time ($F = 116.89$; $p < 0.001$), two way repeated measures ANOVA and Tukey test. C – The nociception induced by a low dose of capsaicin (third bar) was significantly increased by pre-treatment (three hours before) with carrageenan (fourth bar), as indicated by the symbol "*". This increase was blocked by the selective TRPA1 antagonist HC 030031 (fifth bar), which did not affect capsaicin-induced nociceptive behavior by itself (sixth bar). The symbols "+" indicate a response significantly greater (Tukey test, $p < 0.05$) than that of control groups (first and second bars). Abbreviations: vehicle = 0.9% NaCl + DMSO (dimetilsulfoxide); HC = HC 030031, selective TRPA1 antagonist; Cg = carrageenan. In these and in subsequent figures, numbers between parentheses indicate the number of animals in each group. See Methods for additional details regarding data presentation and analysis.

animal experimental procedures and protocols were approved by the Committee on Animal Research of the State University of Campinas. The animals were housed in plastic cages with soft bedding (five/cage) on a 12-h light/dark cycle (lights on at 6:00 AM) in a temperature-controlled (±23 °C) vivarium. Food and water were available *ad libitum*.

2.2. Drugs

Carrageenan (50, 100 or 300 μg) and capsaicin (0.5 μg) were obtained from Sigma–Aldrich (St. Louis, MO, USA), the TRPA1 antagonist HC 030031 (300 or 1200 μg) was obtained from Tocris Bioscience (Bristol, UK, USA). Carrageenan was freshly prepared and dissolved in 0.9% NaCl (sterile saline), capsaicin was dissolved in 0.9% NaCl and HC 030031 in dimethyl sulfoxide (DMSO). All drugs were injected in the subcutaneous tissue of the plantar surface of the rat's hind paw.

2.3. Behavioral tests

Testing sessions took place during light phase (between 09:00 AM and 5:00 PM) in a quiet room maintained at 23 °C. Before the experiments, each animal was manipulated for 7 days to be habituated to the experimental manipulation. On the day of the experiment, each animal was individually placed in the test chamber 15 min before the beginning of the tests to minimize stress. Rats did not have access to food or water during the test and each animal was used once.

2.3.1. Mechanical hyperalgesia

The test chamber consists of acrylic cages (12 × 20 × 17 cm high) with a wire grid floor. A tilted mirror below the grid provided a clear view of the animal's hind paw. Before testing, the animals were quiet, without exploratory movements and not resting over the paws. The test consisted of evoking a hind paw flexion reflex with a handheld force transducer (electronic anesthesiometer, IITC Life Science, Woodland Hills, CA, USA) fitted with a 0.5 mm^2 polypropylene tip. The tip is applied to the central area of the plantar hind paw with a gradual increase in pressure. After paw withdrawal, the intensity of the pressure was automatically recorded. The test was performed at each defined time intervals, for example, for Fig. 1A at 0, 0.5, 1.0, 2.0 3.0, 6.0 and 24 h after injection, during test intervals animals returned to their house-cages. The baseline paw-withdrawal threshold was defined as the mean of three tests performed before intraplantar injection, being 60 g the upper limit pressure. The nociceptive threshold is defined as the mean of three tests performed, at each time interval after intraplantar injection. Mechanical hyperalgesia was quantified as the change in the mechanical nociceptive threshold, expressed by the delta (Δ) withdrawal threshold (in g) calculated by subtracting the nociceptive threshold, at each indicated times after injection, from the baseline threshold.

2.3.2. Overt nociception

The test chamber consists of a mirrored-wood chamber (30 × 30 × 30 cm) with a glass at the front side. Following the intraplantar injection, the behavioral nociceptive response was quantified by counting the number of spontaneous flinches/shakes of the injected hind paw during 5 min.

2.4. Measurement of myeloperoxidase activity

Myeloperoxidase is one of the enzymes released from neutrophils and directly associated to tissue injury. Although monocites/macrophages (Klebanoff, 1980) and fibroblasts also contain myeloperoxidase (Nozawa-Inoue et al., 2003), neutrophils show the highest intracellular levels of this enzyme, that represents up to 5% of neutrophil proteins (Klebanoff, 1991).

The myeloperoxidase kinetic-colorimetric assay was used, as previously described (Torres-Chavez et al., 2012). Three hours after injection, the tissue of the plantar surface of the hind paw was dissected into a standard sized sample, weighed, quickly frozen by liquid isopentane in dry ice and stored at −70 °C. In the assay procedure, each sample was homogenized in 500 μl of buffer 1 (0.1 M NaCl, 0.02 M $NaPO_4$, 1.015 M Na EDTA, pH 4.7) followed by centrifugation at 3000 rpm for 15 min. The pellet was resuspended in 500 μl of buffer 1 and subjected to hypotonic lyses by the addition of 500 μl of 0.2% NaCl followed 30 s later by addition of 500 μl of 1.6% NaCl in 5% glucose. After a further centrifugation, the pellet was resuspended in 0.05 M $NaPO_4$ buffer (pH 5.4) containing 0.5% hexadecyl-trimethylammonium bromide (HTAB). After that, the samples were snap-frozen in liquid nitrogen and thawed, three times, and centrifuged at 10,000 rpm for 15 min. Fifty microliters of each sample (supernatant) and of 0.08 M $NaPO_4$ were dropped into wells of

a 96-well microplate. 25 μl of 3,3′, 3,3′-tetramethylbenzidine was added in each well and the reaction was initiated by the addition of 100 μl of H_2O_2. The reaction was stopped 5 min later by the addition of 50 μl of 4 M H_2SO_4. The optical density was read at 450 nm using an Anthos 2020. Results were calculated by comparing the optical density of hind paw tissue supernatant with a standard curve of neutrophil (>95% purity) obtained as previously described (Torres-Chavez et al., 2012). The results were presented as number of neutrophils $\times 10^6$/mg tissue.

2.5. Cytokines measurement

Three hours after injection, the tissue of the plantar surface of the hind paw was dissected into a standard sized sample, weighed, snap frozen on dry ice and store at −70 °C. The time-point for cytokines measurement was defined based on the time course of carrageenan-induced inflammatory hyperalgesia. Cytokines measurement was performed as previously described (Torres-Chavez et al., 2011). Prior to the assay, hind paw tissue was homogenized in phosphate buffered saline (PBS, pH = 7.4) containing 0.4 M NaCl, 0.05% Tween-20, 0.5% bovine serum albumin, 0.1 mM phenylmethylsulfonyl fluoride, 0.1 M benzethonium chloride, 10 mM EDTA and 20 kl/mL aprotinin. The homogenenates were centrifuged at 12,000 RPM for 15 min at 4 °C. Two-site ELISA was used to measure the cytokines TNF-α, IL1-β and IL-6 using an immunoassay kit (DuoSet, R&D) as described by the manufacturer. The results are expressed as pictograms (pg) of each cytokine per gram (g) of hind paw tissue.

2.6. Antisense oligodeoxynucleotides (ODNs)

The functional blockade of TRPA1 receptor expression on peripheral sensory neurons was performed by the intrathecal injection of antisense ODN. The following antisense ODN sequence was used: 5′-TATCGCTCCACATTGCTAC-3′ The mismatch-ODN sequence, 5′-ATTCGCCTCACATTGTCAC-3′, corresponded to the antisense sequence except that six bases were changed. A search on the NCBI database to *Rattus norvegicus* identified no other sequences homologous to that used in this experiment. The antisense ODNs were synthesized by Erviegas (São Paulo, SP, Brazil), lyophilized and reconstituted in 0.9% NaCl.

Antisense ODN was intrathecally injected as previously described (Papir-Kricheli et al., 1987). Briefly, for each injection, rats were anesthetized with 1/3 O_2 to 2/3 N_2O and halothane at 5% and 1.5%, respectively. A 26-gauge needle was inserted in the subarachnoid space on the midline between L5 and L6 vertebrae. The animals regained consciousness approximately 1 min after discontinuing the anesthetic. A dose of 5 nmol of TRPA1 antisense- or mismatch-ODN (Andrade et al., 2008) was intrathecally administered in a volume of 10 μl (at 1 μl/s) once daily for 4 days. Experiments were initiated 1 h after the last injection.

2.7. Western-blot analysis of TRPA1 expression

The immunoblot study was conducted as previously described (Romero-Calvo et al., 2010) to assess the efficacy of antisense-ODN treatment and to quantify TRPA1 expression after carrageenan injection. Previously stored L5-L6 DRG samples were homogenized with an ultrasonic homogenizer (Sonic Corporation, USA), in a buffer containing 1% Triton X-100, 50 mM Phosphate Buffer, pH 7.4, 8 M Urea, 2 M thiourea, 1 mM EDTA, 1% complete protease inhibitor cocktail (Sigma, USA – P8340), at 4 °C. After 20 min incubation at 4 °C, the samples were centrifuged at 12,000 *g* for 15 min at 4 °C, and the resulting supernatant was transferred to a new tube. Protein concentration was determined by the Bradford method, and 70 μg of total proteins from each sample were separated by SDS-PAGE and transferred to nitrocellulose membranes according to standard techniques. Membranes were stained with Ponceau S, and digital images were acquired for the control of protein loading by densitometric analysis (Romero-Calvo et al., 2010). Membranes were blocked with 5% non-fat dry milk solution in PBS containing 0.1% tween-20, and incubated overnight with anti-TRPA1 (1:1000), followed by HRP conjugated secondary antibody (Zymed, 1:10,000). Reactive bands were detected with the Super Signal West Pico chemiluminescent kit. Results are expressed as the ratio between TRPA1 and the corresponding Ponceau S optical densities.

2.8. Statistical analysis

A two-way repeated measures ANOVA with one between-subjects factor (i.e., treatment) and one within-subjects factor (i.e., time) was used to determine whether there were significant differences among the groups in Fig. 1A,B and Fig. 3A. To determine if there were significant differences between treatment groups in Figs. 1C and 2 one-way ANOVA was performed. If there was a significant between-subjects main effect of treatment group following ANOVA, post hoc contrasts using the Tukey test were performed to determine the basis of the significant difference. To determine if there were significant differences between treatment groups in Fig. 3B and C a *t*-test was performed. The level of significance was set at $p < 0.05$. Data are expressed in figures by intensity of mechanical hyperalgesia (Fig. 1A, B and Fig. 3A), by number of paw flinches (Fig. 1C), by amount of cytokines (pg) per gram of tissue (Fig. 2A, B and C), by the number of neutrophils per gram of tissue (Fig. 2D) or by TRPA1 expression (relative optical density, Fig. 3B and C) and are presented as means ± S.E.M.

3. Results

3.1. Carrageenan-induced inflammatory hyperalgesia

The subcutaneous administration of carrageenan into the plantar surface of the rat hind paw significantly reduced the mechanical paw withdraw threshold, referred as hyperalgesia, in a dose and time-dependent manner. The hyperalgesic effect peaked at 3 h and was still present at 24 h after the carrageenan administration. The 100 μg dose of carrageenan induced a mechanical hyperalgesia similar in magnitude to that induced by 300 μg and significantly higher than that induced by 50 μg (Fig. 1A). Therefore, the 100 μg dose was used in further experiments. Two-way repeated-measures ANOVA showed a significant main effect of treatment ($F = 567.60$; $p < 0.001$) and a significant treatment × time interaction ($F = 38.68$; $p < 0.001$).

3.2. Effect of pharmacological blockade of TRPA1 on carrageenan-induced inflammatory hyperalgesia

To evaluate the contribution of TRPA1 to carrageenan-induced mechanical inflammatory hyperalgesia, TRPA1 was pharmacologically blocked by its selective antagonist HC 030031. HC 030031 was either co-administrated with carrageenan or administrated 2 h and 55 min after the carrageenan administration, that is, 5 min before the time at which carrageenan-induced hyperalgesia is maximal. The co-administration of HC 030031 (at 1200 μg, but not at 300 μg) significantly reduced carrageenan-induced mechanical hyperalgesia at 3 but not 6 h after its administration (Fig. 1B). The post-administration of HC 030031 (1200 μg) significantly reduced carrageenan-induced mechanical hyperalgesia at 3 and 6 h after the carrageenan administration (Fig. 1B). Two-way repeated-measures ANOVA showed a significant main effect of treatment ($F = 600.37$; $p < 0.001$) and a significant treatment × time interaction ($F = 199.67$; $p < 0.001$).

The administration of HC 030031 (1200 μg) into the contralateral hind paw had no effect, indicating that the anti-hyperalgesic effect of HC 030031 is mediated by the blockade of local TRPA1 (carrageenan in the ipsilateral plus HC 030031, 32.22 g ± 0.75, or its vehicle, 32.26 g ± 0.87, in the contralateral hind paw, *t* test, $p = 0.97$). HC 030031 did not affect the mechanical threshold evaluated three hours after the intraplantar saline (0.9% NaCl) administration (0.9% NaCl plus HC 030031, 0.71 g ± 0.11, or its vehicle, 0.40 g ± 0.24, data not shown, *t* test, $p = 0.30$).

To further demonstrate the involvement of TRPA1 in carrageenan-induced inflammatory hyperalgesia, we used a chemical rather than a mechanical stimulus to detect carrageenan-induced inflammatory hyperalgesia. The chemical stimulus, a low dose of capsaicin (0.5 μg) that induces minimal nociceptive behavior, was applied 3 h after the intraplantar administration of carrageenan at the same local. Under this condition, capsaicin-induced behavioral nociceptive response was significantly increased (Fig. 1C) and used as a quantitative measurement of carrageenan-induced hyperalgesia. The co-administration of the TRPA1 receptor antagonist HC 030031 (1200 μg) with carrageenan blocked carrageenan-induced increase in capsaicin-induced nociceptive behavior, without affecting capsaicin-induced nociceptive behavior by itself (Fig. 1C, one way ANOVA and Tukey test, $p = <0.001$). These findings corroborate the involvement of TRPA1 in carrageenan-induced inflammatory hyperalgesia.

3.3. Effect of pharmacological blockade of TRPA1 on carrageenan-induced cytokines expression and neutrophil migration

The co-administration of the selective TRPA1 antagonist HC 030031 (1200 μg) did not affect carrageenan-induced either the expression of the cytokines TNF-alpha (Fig. 2A, Tukey test,

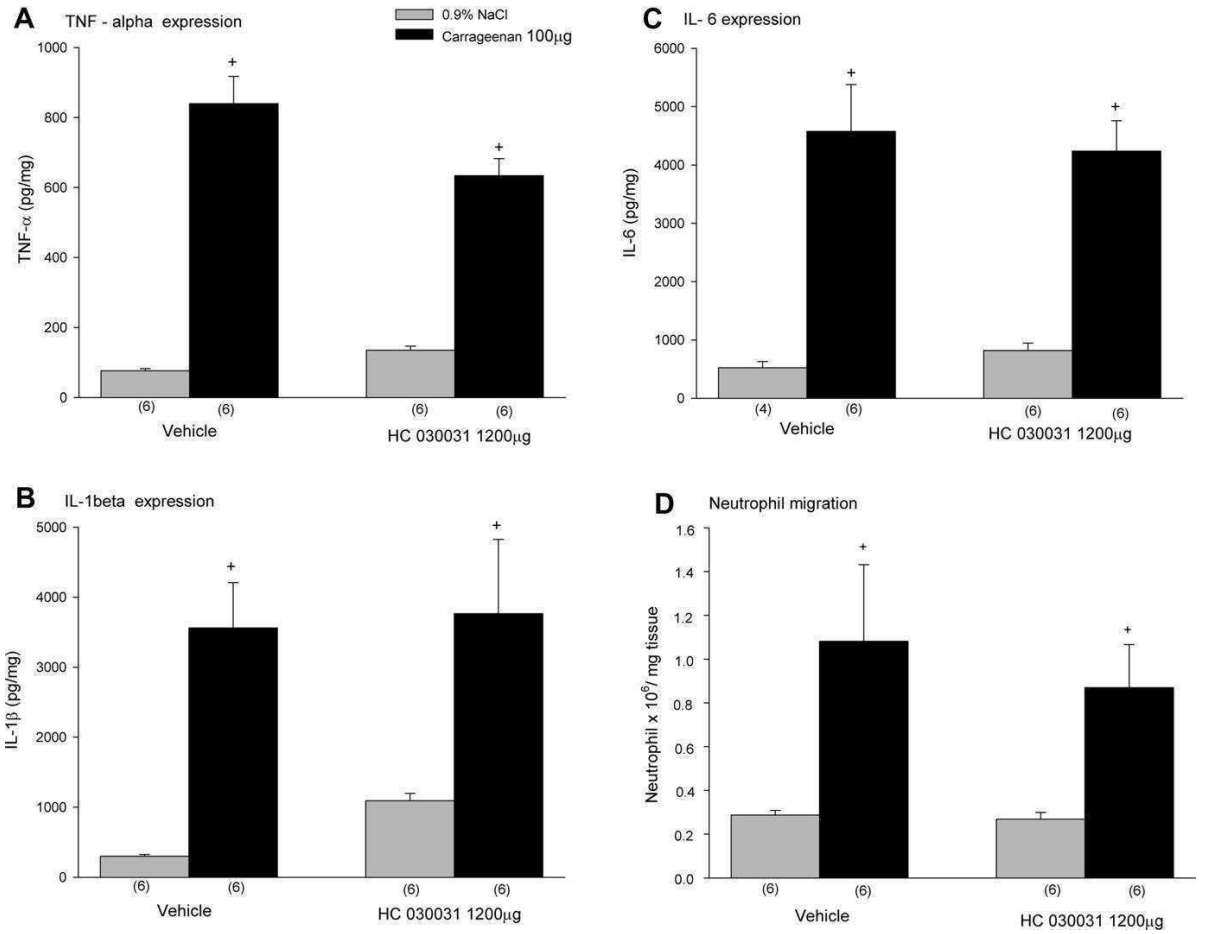

Fig. 2. Effect of pharmacological blockade of TRPA1 on carrageenan-induced cytokines expression and neutrophil migration. The pharmacological blockade of TRPA1 by the co-administration of its selective antagonist HC 030031 with carrageenan did not affect carrageenan-induced TNF-alfa (A), IL-1 beta (B), IL-6 (C) expression and neutrophil migration (D) (One Way Anova and Tukey test, $p > 0.05$). The symbols "+" indicate a response significantly greater than that induced by 0.9% NaCl (t test, $p < 0.05$).

$p > 0.05$), IL-1 beta (Fig. 2B, Tukey test, $p > 0.05$) and IL-6 (Fig. 2C, Tukey test, $p > 0.05$) or neutrophil migration (Fig. 2D, Tukey test, $p > 0.05$).

3.4. Contribution of neuronal TRPA1 to carrageenan-induced hyperalgesia

TRPA1 gene silencing by pre-treatment with antisense-ODN but not with mismatch-ODN (daily intrathecal injection, between L5 and 6, of 5 nmol for 4 days) against TRPA1 prevented carrageenan-induced mechanical inflammatory hyperalgesia (Fig. 3A). Two-way repeated-measures ANOVA showed a significant main effect of treatment ($F = 769.33$; $p < 0.001$) and a significant treatment × time interaction ($F = 511.82$; $p < 0.001$). TRPA1 gene silencing by pre-treatment with antisense-ODN did not affect (0.26 g ± 0.06) the mechanical paw withdraw threshold (0.40 g ± 0.25) evaluated three hours after the subcutaneous saline (0.9% NaCl) injection in the hind paw (t test, $p = 0.58$, data not shown).

Treatment with antisense-ODN significantly decreased TRPA1 expression in DRG of L5 and L6 (Fig. 3B, t test $p < 0.001$), which provide the neural supply to the plantar surface of the hind paw. The subcutaneous administration of carrageenan into the hind paw did not significantly increase TRPA1 expression in the DRG (L5-6) (Fig. 3C, t test, $p > 0.05$).

4. Discussion

In this study, we showed that neuronal TRPA1 plays an important role in the development and maintenance of carrageenan-induced inflammatory hyperalgesia by directly contributing to nociceptor sensitization.

The selective TRPA1 antagonist HC 030031 prevented and reversed carrageenan-induced mechanical hyperalgesia indicating that TRPA1 is necessary for the development and maintenance of the inflammatory hyperalgesia. The anti-hyperalgesic effect of HC 030031 lasted up to three hours, which is consistent with the previously reported duration of its effect (da Costa et al., 2010). When the effect of HC 030031 disappeared, the hyperalgesic response returned, suggesting that a sustained TRPA1 activation is critical for the maintenance of nociceptor sensitization induced by the inflammatory response. This suggestion is further supported by previous *in vivo* studies showing that TRPA1 mediates long-lasting hyperalgesic responses in different models (da Costa et al., 2010;

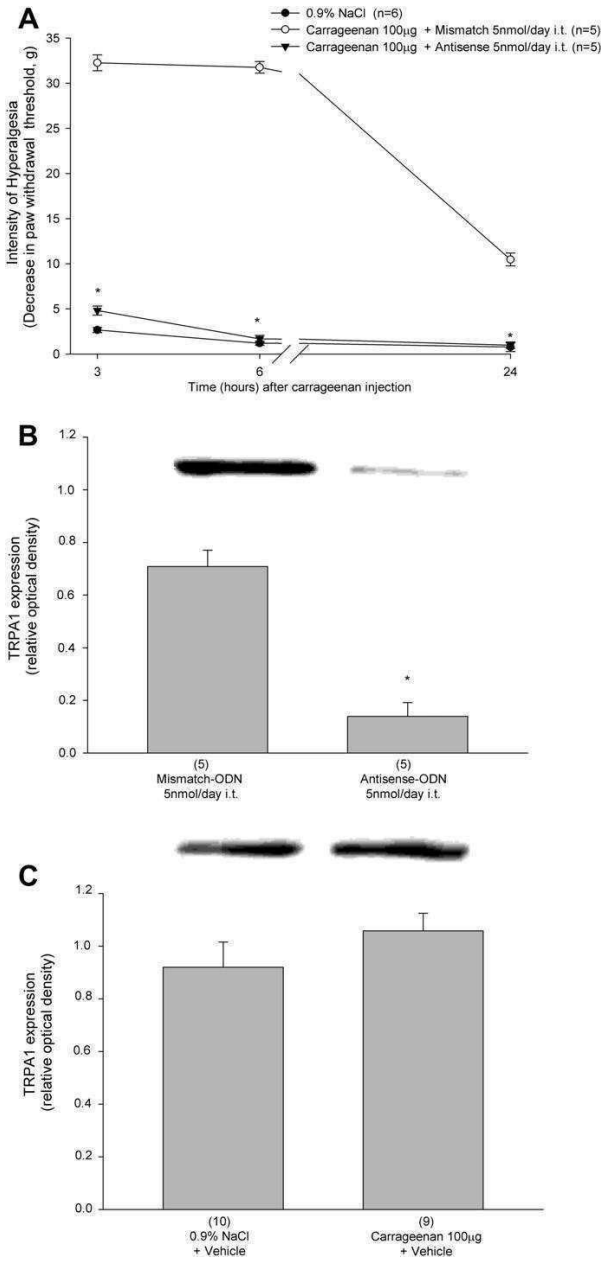

Fig. 3. Direct contribution of TRPA1 to carrageenan-induced inflammatory hyperalgesia. A – TRPA1 gene silencing by antisense-ODN (5 nmol daily, intrathecal injection, for four days) prevented carrageenan-induced mechanical inflammatory hyperalgesia, as indicated by the symbols "*". There is a statistically significant interaction between treatment and time ($F = 511.82$; $p < 0.001$), two way repeated measures ANOVA and Tukey test. B – Treatment with antisense-ODN significantly decreased TRPA1 expression in the dorsal root ganglia (L5 and L6) cells, as indicated by the symbol "*" (t test, $p = <0.001$). C – Intraplantar injection of carrageenan did not significantly increase TRPA1 expression in the dorsal root ganglia (L5 and L6), t test, $p > 0.05$. Abbreviations: i.t. = intrathecal; vehicle = 0.9% NaCl + DMSO (dimetilsulfoxide); HC = HC 030031 (selective TRPA1 antagonist).

Dunham et al., 2008; Eid et al., 2008; Obata et al., 2005; Petrus et al., 2007) and by *in vitro* studies showing sustained TRPA1 activation using the perforated patch clamp technique (Koivisto et al., 2012) and intracellular calcium images (Koivisto et al., 2012).

The involvement of TRPA1 in the mechanical transduction in dorsal root ganglia neurons is unclear (Bhattacharya et al., 2008; Kerstein et al., 2009; Rugiero and Wood, 2009). However, here we demonstrated that the pharmacological blockade of TRPA1 or its gene silencing did not affect the mechanical paw withdraw threshold, arguing against a role for this receptor in nociceptor mechanical transduction. Furthermore, the blockade of TRPA1 significantly reduced carrageenan-induced inflammatory hyperalgesia detected also by a chemical stimulus (low dose of capsaicin), further demonstrating the involvement of TRPA1 in carrageenan-induced inflammatory hyperalgesia.

The carrageenan administration is a classical animal model used to screen drugs for inflammatory pain treatment and control. It induces a widespread inflammatory reaction including edema, cytokines release and neutrophil migration (Ferreira et al., 1993; Winter et al., 1962). We showed that the selective TRPA1 antagonist HC 030031 at a dose that significantly reduced the inflammatory hyperalgesia did not affect cytokines expression and neutrophil migration induced by carrageenan. Therefore, TRPA1 contributes to edema formation (Moilanen et al., 2012) but not to cytokines release or neutrophil migration induced by carrageenan. Taken together, these findings suggest that the role of TRPA1 in carrageenan-induced inflammation may be restricted to edema formation, which is the major consequence of neurogenic inflammation. Although the activation of TRPA1 in nociceptive neurons may contribute to carrageenan-induced cytokines expression and neutrophil migration through neuropeptides release, this contribution is expected to be weak. This is because the major inflammatory function of endogenous neuropeptides is to regulate vascular permeability, although the exogenous administration of neuropeptides can induce cytokines expression and neutrophil migration (Ansel et al., 1993; Nakagawa et al., 1995). Despite of its well known role in neurogenic inflammatory mechanisms, the expression of TRPA1 in non-neuronal cells and the more recent evidences that its activation in these cells leads to the release of cytokines (Atoyan et al., 2009) and the formation of prostaglandins and other inflammatory mediators (Jain et al., 2011) culminate with the idea that TRPA1 may regulate multiple inflammatory mechanisms (Fernandes et al., 2012). However, our data indicate that the important role played by TRPA1 in carrageenan-induced inflammatory hyperalgesia does not depend on cytokines release and neutrophil migration.

The ability of neuronal TRPA1 gene silencing in completely preventing carrageenan-induced hyperalgesia, indicates that the important role of TRPA1 in carrageenan-induced inflammatory hyperalgesia is mediated by a direct contribution to nociceptor excitability, via neuronal TRPA1 activation. The neuronal TRPA1 gene silencing was induced by intrathecal pre-treatment with antisense-ODN against TRPA1 which strongly reduced TRPA1 expression in the dorsal root ganglia cells (L5-6). While TRPA1 located on the peripheral endings of primary afferent nociceptors contributes to inflammatory sensitization (da Costa et al., 2010; Dunham et al., 2008; Eid et al., 2008; Obata et al., 2005; Petrus et al., 2007), those located on central endings facilitate the transmission of nociceptive information to the second order neuron in the spinal dorsal horn (da Costa et al., 2010; Kosugi et al., 2007; Wei et al., 2011). Therefore, the ability of the antisense-ODN in completely preventing carrageenan-induced hyperalgesia probably results from the blockade of central, in addition to peripheral TRPA1-mediated mechanisms.

The mechanisms underlying the sustained TRPA1 activation in response to carrageenan are presently unknown, but may involve bradykinin release which indirectly gate TRPA1. In addition, carrageenan might induce endogenous TRPA1 agonists release, because many of the oxidants produced during inflammation, such as nitro-

oleic acid (Taylor-Clark et al., 2009), 4-hydroxynonenal (Trevisani et al., 2007) or hydrogen peroxide (Andersson et al., 2008) directly gate TRPA1. Therefore, many endogenously produced substances during the inflammatory response triggered by carrageenan may induce TRPA1 activation. Prostaglandin E_2 and sympathetic amines are the most important primary mediators ultimately responsible for inflammatory sensitization (Khasar et al., 1999; Nakamura and Ferreira, 1987). They increase neuronal membrane excitability by activating signaling pathways (cyclic adenosine monophosphate/protein kinase A (PKA) and phospholipase C (PLC)) that also activate TRPA1 (Wang et al., 2008). Therefore, further studies are warranted to evaluate whether these mediators contributes to TRPA1 activation in the carrageenan model. However, unpublished observations from our lab suggest that Prostaglandin E_2 activates TRPA1 through a PKA dependent mechanism.

In contrast to nerve injury (Frederick et al., 2007; Obata et al., 2005) and the Freund's complete adjuvant (CFA)-induced inflammation (da Costa et al., 2010; Dunham et al., 2008; Obata et al., 2005), carrageenan-induced inflammation did not increase TRPA1 expression in dorsal root ganglia cells of L5 and L6. However, nerve injury and CFA induce long-lasting biochemical changes that strongly modulate gene expression in peripheral nerves, while carrageenan induces a widespread but shorter-lasting inflammatory process, which is maximal at three and almost resolved at twenty four hours. However, it is possible that carrageenan increases the expression of functional TRPA1 in the cell membrane rather than TRPA1 gene expression. Consistent to this idea, it was demonstrated that TRPA1 membrane insertion is mediated by PKA and PLC (Schmidt et al., 2009), both of which are involved in carrageenan-induced hyperalgesia.

In summary, we showed that TRPA1 plays an important role in the development and maintenance of carrageenan-induced inflammatory hyperalgesia, its neuronal activation directly contributes to nociceptor sensitization. Therefore, a sustained TRPA1 activation seems to be critical for the maintenance of nociceptor sensitization during carrageenan-induced inflammatory hyperalgesia. Based on these findings, we suggest that TRPA1 blockade is a promising strategy for the development of future drugs to pain treatment and control.

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