Pontifícia Universidade Católica do Rio Grande do Sul
Programa de Pós-Graduação em Medicina e Ciências da Saúde
Área de Concentração: Farmacologia Bioquímica e Molecular

Jerônimo Pietrobon Martins

PARTICIPAÇÃO DOS RECEPTORES PURINÉRGICOS P2X7 NA CISTITE HEMORRÁGICA INDUZIDA PELA CICLOFOSFAMIDA EM CAMUNDONGOS

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Dissertação apresentada como requisito parcial para obtenção do Título de Mestre pelo Programa de Pós-Graduação em Medicina e Ciências da Saúde, Área de Concentração em Farmacologia Bioquímica e Molecular, da Pontifícia Universidade Católica do Rio Grande do Sul.

Orientador: Profª. Dra. Maria Martha Campos

Co-Orientador: Profª. Dra. Fernanda Bueno Morrone

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Aprovada em _______ de _____________________ de __________

BANCA EXAMINADORA

Dr. Rafael Fernandes Zanin - UFRGS

____

Dra. Carla Denise Bonan - PUCRS

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Dr. Maurício Reis Bogo - PUCRS

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*Dedico este trabalho a minha família
e a minha namorada por todo seu
amor e carinho e por estarem sempre
comigo apoiando minha jornada.*

AGRADECIMENTOS

À minha orientadora, Profª Dra. Maria Martha Campos por me disponibilizar esta oportunidade de alcançar um sonho. Pela serenidade com que me passou seus conhecimentos, por seu carinho e por suas críticas e sugestões.

À minha co-orientadora Profª Dra Fernanda Bueno Morrone por me apoiar e incentivar a entrar e seguir na pesquisa científica desde a graduação, por seus ensinamentos e pela amizade.

Ao colega de laboratório e amigo Rodrigo Braccini por ter contribuído com o desenvolvimento deste trabalho, por se mostrar sempre disponível a ajudar e por sua amizade.

Aos colegas e amigos do laboratório de farmacologia, incluindo o técnico Juliano Soares por sua amizade e companheirismo. E ao André Avelino dos Santos Júnior e Vinicius Boeira por suas contribuições com o trabalho.

Ao Tiago técnico do laboratório de patologia e as farmacêuticas e técnicas da quimioterapia por suas contribuições para com este estudo.

Ao Dr. Robson Coutinho pela receptividade e contribuições com o trabalho.

À Profº Dra. Christina Maeda Takiya pela receptividade em seu laboratório, ensinamentos e por fazer com que o pouco tempo disponível para realização dos experimentos gerasse bons frutos. Aos amigos do laboratório da Profª Christina da UFRJ, Vivian, Luis e Deisi por me auxiliarem no desenvolvimento dos experimentos.

Ao meu amigo Alexandre por me acolher em sua casa durante o período em que estive no Rio de Janeiro.

Ao Sr. Gilberto Júnior, diretor da empresa Globo Vac, por ter disponibilizado o tempo necessário para a realização do mestrado. E aos meus colegas de trabalho, em especial a Flávia, Ana Mara e Eduardo Roberto por me auxiliarem nos momentos de ausência.

Aos meus amigos Marcos, Bitello, Lúcio, Frederico e Ricardo, Lorenzo pela amizade e incentivo.

A minha segunda família, Ronaldo, Marisa, Ivone e Bruno pelo apoio e pela torcida.

A Simone e a Natalie pelo apoio e pela torcida.

À minha família por ter me incentivado desde o principio e por me fazerem forte nos momentos de fraqueza. À minha mãe e ao meu pai por serem exemplos a serem seguidos na busca do conhecimento, pelo apoio e pela compreensão da minha ausência em determinados

momentos, ensinamentos e conselhos. Aos meus irmãos Gabriel e Fernanda pelo estímulo, amizade e críticas, por serem grandes amigos e por estarem junto comigo durante toda a minha caminhada.

E finalmente a Raquel, minha namorada, amiga e conselheira pelo carinho de sempre, companheirismo, apoio e incentivo. Por compartilhar comigo meus momentos de alegria e por me levantar nos momentos de tristeza. Por estar sempre ao meu lado e por ser um exemplo de profissional. Obrigado por me fazer acreditar em mim.

RESUMO

Nucleotídeos extracelulares são moléculas sinalizadoras importantes que medeiam diversos efeitos biológicos, através da ativação de receptores purinérgicos. O ATP é liberado em resposta ao dano celular e os receptores P2X7 têm um papel essencial no início e na manutenção das várias alterações patológicas. A cistite hemorrágica (CH) é um efeito adverso bem conhecido da terapia com ciclofosfamida (CYP), observada em pacientes sob tratamento de muitos tumores sólidos e doenças auto-imunes. Este trabalho teve como objetivo determinar o papel dos receptores P2X7 no modelo de CH induzida por CYP em camundongos. Os efeitos do antagonismo farmacológico ou ausência gênica do receptor P2X7 na CH induzida pela CYP foram avaliados em uma série de parâmetros nociceptivos e inflamatórios. Além disso, a imunopositividade para o receptor P2X7 foi investigada através de análise imunoistoquímica. O pré-tratamento com o antagonista seletivo do receptor P2X7 (A438079) ou a deleção gênica do receptor P2X7 reduziu os escores indicativos de comportamento nociceptivo. As mesmas estratégias produziram uma redução significante dos índices de edema e hemorragia, como indicado pela avaliação macroscópica e histológica. O tratamento com o A438079 também diminuiu significativamente a marcação positiva para c-Fos na medula espinhal e em áreas corticais do cérebro. Notavelmente, a administração do A438079 preveniu o aumento da atividade de MPO, da migração de macrófagos e dos níveis de IL-1β e TNFα no tecido vesical de animais que receberam CYP. Por fim, foi detectado um aumento significativo do receptor P2X7 na bexiga de animais tratados com CYP. O presente estudo revelou a importância do receptor P2X7 na CH induzida pela CYP. A inibição farmacológica deste receptor poderia representar uma nova alternativa terapêutica para esta condição patológica ou, ainda, em outros tipos de cistite sem origem definida.

Palavras-chave: receptor P2X7; cistite hemorrágica; ciclofosfamida; A438079; deleção gênica; camundongos.

ABSTRACT

Extracellular nucleotides are important signaling molecules that mediate many biological effects, through the purinergic receptor activation. ATP is released in response to cellular damage, and P2X7 receptors have an essential role in the onset and maintenance of pathological changes. Haemorrhagic cystitis (HC) is a well known adverse effect of therapy with cyclophosphamide (CYP) used in patients under the treatment of many solid tumours and autoimmune conditions. This study aimed to determine the role of P2X7 receptors in the mouse model of HC induced by CYP. The effects of pharmacological antagonism or genic absence of P2X7 receptor in CYP-induced HC was assessed in a series of nociceptive and inflammatory parameters. In addition the immunopositivity for P2X7 receptors was investigated by means of immunohistochemistry. The pre-treatment with the selective P2X7 receptor antagonist A438079 or genic ablation of P2X7 receptors inhibited the nociceptive behaviour score induced by CYP. The same strategies produced a significant reduction of both oedema and haemorrhage indexes, as indicated by either macroscopic or histological evaluation. A438079 treatment also significantly decreased the positive staining for c-Fos in the lumbar spinal cord and brain cortical areas. Noteworthy, the administration of A438079 markedly prevented the increase of urinary bladder MPO activity and macrophage migration induced by CYP, and widely reduced the tissue levels of IL-1β and TNFα. Finally, P2X7 receptor was found strikingly up-regulated in the bladders of CYP-treated mice. The present study revealed the importance of P2X7 receptors in HC induced by CYP. The pharmacological inhibition of these receptors might represent a new therapeutic alternative for this pathological condition.

Keywords: P2X7 receptor; haemorrhagic cystitis; cyclophosphamide; A438079; genic ablation, mice.

ABREVIAÇÕES

ADP – Adenosina difosfato

AMPc – Adenosina monofosfato cíclico

ATP – Adenosina trifosfato

A438079 – 3-[[5-(2,3-diclorofenil)-1*H*-tetrazol-1-yl]metil]cloridrato de piridina

BzATP – trifosfato de adenosina benzoíla

CH – Cistite Hemorrágica

COX-2 – Ciclo-oxigenase 2

CYP – Ciclofosfamida

HTAB – Brometo de Amônio Hexadeciltrimetil

IL-1β – Interleucina 1beta

iNOS – Óxido Nítrico Sintase Induzível

KO – Knockout

Mesna – 2-mercaptoetanosulfonato de sódio

NO – Óxido Nítrico

NOS – Óxido Nítrico Sintase

PAF – Fator de Ativação Plaquetária

PBS – Solução Tampão fosfato salina

PPADS – Piridoxal-fosfato-6-azofenil-2’,4’-dissulfonato

TBS – Solução Tampão Salina Tris

TGF-β – Fator de transformação do crescimento beta

TNF-α – Fator de Necrose Tumoral alfa

UDP – Uridina difosfato

UTP – Uridina trifosfato

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1. Introdução

A cistite hemorrágica (CH) é uma alteração da mucosa da bexiga, caracterizada pela presença de hematúria e sintomas de irritabilidade, tais como disúria, freqüência e urgência na micção (Traxer et al., 2001; Nickel, 2002; Cheuk et al., 2007; Manikandan et al., 2010). Pode ser causada por agentes químicos ou por radiação. Dentre os agentes químicos que causam a CH estão as oxafosforinas (ciclofosfamida e ifosfamida), e o busulfano, além de outros medicamentos como as penicilinas e os antiinflamatórios não esteroidais, que podem ocasionalmente gerar CH. A CH também pode ser observada após a radioterapia para o tratamento de carcinomas próximos à região pélvica. Pode ainda estar relacionada com infecções virais (por poliomavírus, adenovírus ou citomegalovírus), bacterianas, fúngicas ou parasitárias (Traxer et al., 2001; Neheman et al., 2005; Giraud et al., 2006; Cheuk et al., 2007; Manikandan et al., 2010). Muitos casos de CH leve são resolvidos espontaneamente sem complicações; porém casos de CH moderada à severa podem resultar em significante morbidade ou até mesmo mortalidade (Cheuk et al., 2007; Manikandan et al., 2010).

A ciclofosfamida (CYP) é um agente alquilante, que atua de maneira inespecífica no ciclo celular, sendo usada comumente no tratamento de muitos tumores sólidos, neoplasia de células B e em algumas doenças auto-imunes (lupus e artrite reumatóide). Além disso, pode ser usada após transplante de medula óssea, devido a sua potente atividade imunossupressora, sendo administrada por via oral ou intravenosa (Crocitto et al.; 1996, Wong et al.; 2000, Chow et al.; 2006; Brunton et al. 2006; Mesquita et al. 2007).

Quando utilizada como terapia adjuvante para o câncer de mama, bem como para pacientes com linfomas e leucemia linfocítica crônica, a dose recomendada é 100 mg/m^2, durante 14 dias. É também utilizada no tratamento do câncer de mama e dos linfomas em uma dose mais alta, de 500 mg/m^2, por via intravenosa, a cada 2 a 4 semanas, podendo ainda ser associada a outros fármacos. É um componente essencial utilizado em muitas combinações de fármacos, como por exemplo, a associação com metrotrexato e fluorouracila na terapia adjuvante após cirurgia do carcinoma de mama (Brunton et al. 2006).

A CYP é metabolizada no fígado gerando a 4-hidroxiciclofosfamida e, em equilíbrio dinâmico, seu tautômero acíclico, a aldofosfamida. A 4-hidroxifosfamida pode ser oxidada pela aldeído-oxidase no fígado ou no tecido tumoral produzindo os metabólitos inativos

carboxifosfamida e 4-cetociclofosfamida (Figura 1). A aldofosfamida e a 4-hidroxiciclofosfamida, são transportados pela circulação até as células tumorais. Nestas células, a aldofosfamida sofre clivagem produzindo uma mostarda de fosforamida (agente ativo) e a acroleína que é diretamente tóxica para o urotélio (Kranc et al., 1992; Wong et al., 2000; Rang et al. 2004; Korkmaz et al., 2007).

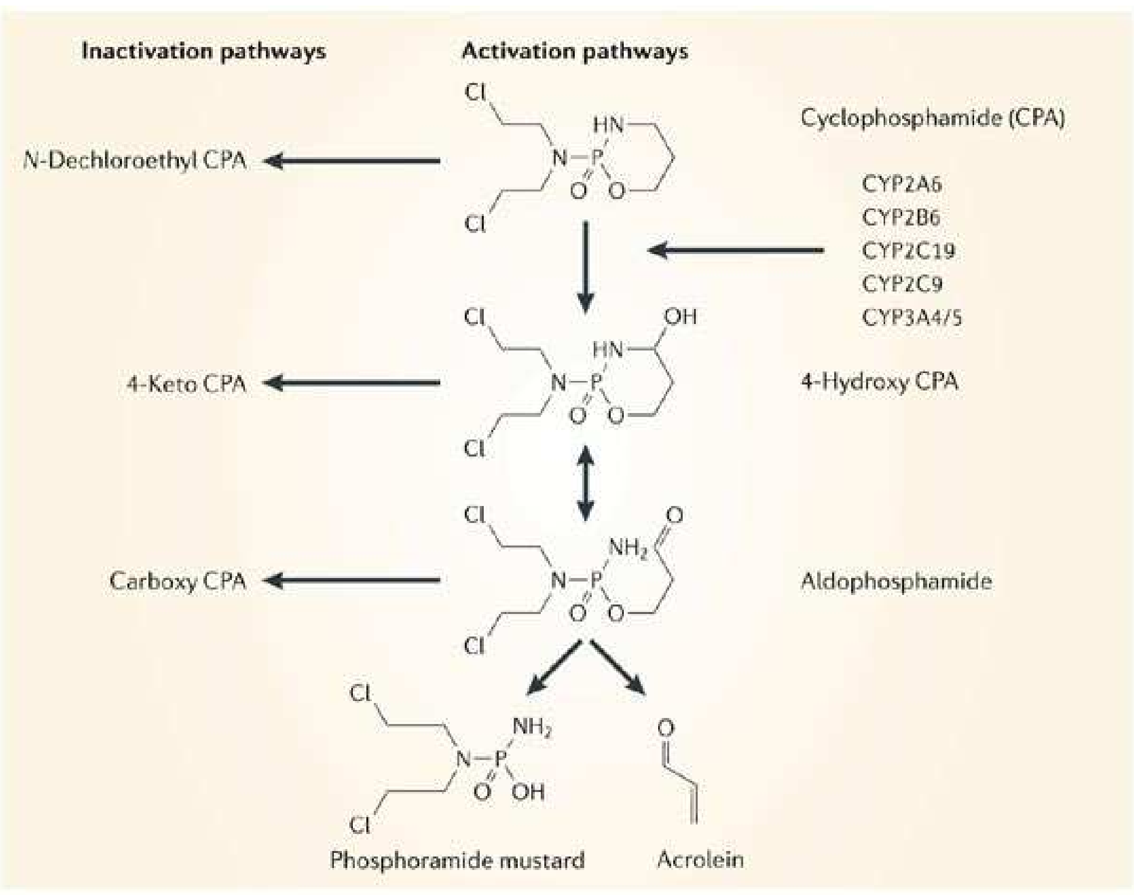

Figura 1. Metabolismo da ciclofosfamida.

Além de ser um metabólito da CYP, a acroleína ocorre naturalmente em alimentos e é formada durante a combustão de materiais orgânicos. Deste modo, a acroleína é encontrada em todos os tipos de fumaça, incluindo fumaça de cigarro (Brunton et al., 2006; Korkmaz et al., 2007).

Um dos efeitos adversos mais comuns da CYP é a urotoxicidade, que pode incluir cistite hemorrágica e fibrose na bexiga (Crocitto et al., 1996; Wong et al., 2000; Manikandan et al., 2010). A incidência de toxicidade é maior com a administração intravenosa, em comparação com a via oral. Ademais, os danos uroepiteliais induzidos pela CYP podem ocorrer em outros órgãos, além da bexiga (Wong et al., 2000; Chow et al. 2006; Chow et al. 2007).

Uma maneira de evitar a cistite hemorrágica é fazer a hidratação do paciente a fim de manter o fluxo urinário, além da irrigação da bexiga, prevenindo deste modo a obstrução do trato urinário (Traxer et al., 2001; Cheuk et al., 2007; Manikandan et al., 2010). Outra conduta é associar à CYP agentes que neutralizam seu metabólito tóxico (acroleína), tal como o 2-mercaptoetanosulfonato de sódio (Mesna). A administração sistêmica de Mesna resulta em detoxicação regional do sistema urinário (Morais et al., 1999). É recomendada uma dose de Mesna 20% da dose da CYP (Manikandan et al., 2010). O Mesna se liga à acroleína e esta interação resulta em um composto inativo, não anulando a atividade antitumoral do fármaco (Crocitto et al., 1996; Morais et al., 1999). Portanto, o Mesna é indicado para prevenir a ocorrência de cistite hemorrágica, embora não seja efetivo quando a lesão já está estabelecida (Morais et al., 1999).

Outros métodos de prevenção e tratamento da CH incluem a instilação intravesical de salina, formalina, nitrato de prata e prostaglandinas, a administração oral de dissulfiram e N-acetilcisteína e a terapia com oxigênio hiperbárico. Medidas mais agressivas envolvem embolização da artéria hipogástrica, cistectomia com conduto ileal e aumento da bexiga (Traxer et al., 2001; Neheman et al., 2005; Chow et al., 2006; Chow et al., 2007; Manikandan et al., 2010).

Citocinas como TNF-α e IL-1 são medidores cruciais envolvidos nos eventos inflamatórios e também em danos ao urotélio. O óxido nítrico (NO) é o medidor final do dano e da hemorragia urotelial na cistite. A indução de óxido nítrico sintase (NOS) na bexiga inflamada parece ser dependente da ativação de TNF-α e do fator de agregação plaquetária (PAF) (Morais et al., 1999). Embora vários estudos tenham sido destinados à determinação dos processos relacionados com a urotoxicidade da CYP, os mecanismos exatos envolvidos nestas alterações ainda não são completamente conhecidos (Manikandan et al., 2010).

Purinas extracelulares (adenosina, ADP, e ATP) e pirimidinas (UDP e UTP) são moléculas sinalizadoras importantes que medeiam diversos efeitos biológicos, através da ativação

de receptores purinérgicos**.** O conceito de que o ATP é uma molécula sinalizadora extracelular, além de estar envolvido no metabolismo celular como uma fonte de energia, levou muito tempo para ser aceito (Ralevic and Burnstock, 1998; Burnstock, 2006e; Burnstock, 2006b; White and Burnstock, 2006; Burnstock, 2007; Burnstock, 2011).

Estudos realizados por Namasivayam e colaboradores (1999) descreveram que o ATP é liberado endogenamente durante a distensão da bexiga (presumivelmente do urotélio), estando diretamente envolvido na ativação mecanossensitiva do nervo pélvico aferente originado da bexiga. Existem várias evidências mostrando que o ATP está envolvido na neurotransmissão eferente, não-adrenérgica e não-colinérgica da bexiga de muitas espécies animais, incluindo o rato (Namasivayam et al., 1999; Tempest et al., 2004; Sun and Chai, 2006; Burnstock, 2011).

Existem duas famílias principais de receptores purinérgicos: receptores de adenosina ou P1 e, os receptores P2, que reconhecem principalmente ATP, ADP, UTP, e UDP. Os receptores P1/Adenosina são acoplados à proteína G e se subdividem em quatro subtipos, A_1, A_{2A}, A_{2B}, e $A_{3,}$ de acordo com a estrutura molecular, bioquímica e caracterização farmacológica. Baseado em diferentes estruturas moleculares e mecanismo de transdução de sinal, os receptores P2 se dividem em duas famílias de receptores: receptores P2X ionotrópicos e P2Y metabotrópicos. Atualmente, sete subtipos de receptores P2X (P2X1 à P2X7) e oito subtipos de receptores P2Y ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$, $P2Y_{14}$) já foram identificados (Ralevic and Burnstock, 1998; Burnstock, 2006b; Burnstock, 2006e; Burnstock, 2006d; Burnstock, 2007; Surprenant and North, 2009). Exemplos do potencial terapêutico dos receptores P2Y incluem: receptores P2Y1 envolvidos na agregação plaquetária e receptores P2Y2, responsáveis pelo aumento do fluxo de íons cloro, sendo alvos para o tratamento de fibrose cística (von Kugelgen and Wetter, 2000).

A Adenosina regula muitas funções fisiológicas nos sistemas cardiovascular, respiratório, renal, imune, e sistema nervoso central e periférico pela ativação de receptores de adenosina ligados à proteína G (receptor P1). Os receptores P1 e P2Y são geralmente expressos nas mesmas células (Ralevic and Burnstock, 1998; Burnstock, 2007). Recentemente, foi demonstrado que a adenosina evoca a hiperpolarização da membrana e o relaxamento nas células do músculo liso da bexiga de cobaias pela ativação de canais de potássio, via adenilato ciclase e elevação de AMPc (Gopalakrishnan et al., 2002).

Durante o processo inflamatório, ATP e adenosina são liberados no sítio de inflamação como resultado do dano celular. Além disto, podem ser transportados ativamente ao meio extracelular sob ativação celular. Plaquetas ativadas e células endoteliais secretam ATP e ADP sob condições de estresse fisiológico (Luttikhuizen et al., 2004; Burnstock, 2006b; Di Virgilio, 2007a). O ATP está envolvido no desenvolvimento da inflamação através de uma combinação de ações, que são: liberação de histamina dos mastócitos, provocando a produção de prostaglandinas; e a produção e liberação de citocinas das células imunes. Ao contrário do ATP, a adenosina tem uma ação antiinflamatória (Burnstock, 2006b; Burnstock, 2006a; Surprenant and North, 2009).

No urotélio normal da bexiga, estão expressos os subtipos de receptores purinérgicos $P2Y_1$, $P2Y_2$ e $P2Y_4$. Sabe-se que estes receptores não apresentam diferenças significativas na distribuição ou densidade no urotélio da bexiga (Birder et al., 2004). Birder e colaboradores (2004) evidenciaram uma redução significativa na expressão do receptor $P2Y_2$ em um modelo de cistite intersticial em felinos. Os receptores P2Y, diferentemente dos receptores P2X, não são expressos na musculatura lisa da bexiga (Birder et al., 2004).

Na bexiga normal, são expressos todos os sete receptores purinérgicos P2X citados anteriormente. O subtipo predominante é $P2X_3$ seguido por P2X2, P2X1, P2X4, P2X5, P2X6, e P2X7. (O'Reilly et al., 2001a; O'Reilly et al., 2001b; Birder et al., 2004) Há também receptores heteromultiméricos tais como: P2X2/3, P2X1/2, P2X1/5, P2X2/6 e P2X4/6 (Burnstock, 2007). Examinando a distribuição dos subtipos de receptores P2X no músculo liso da bexiga urinária foi encontrado que o receptor P2X2 é o subtipo purinérgico predominante, com menor expressão de P2X1 seguido por P2X3 e P2X7. Os subtipos P2X4, P2X5 e P2X6 não foram identificados (Birder et al., 2004). De fato, estudos com *Northern blotting* e hibridização *in situ* mostraram que receptores P2X1 são predominantemente expressos em músculo liso, receptores P2X4 e P2X6 são principalmente expressos no cérebro, enquanto os receptores P2X7 predominam em células do sistema imune. Achados recentes mostram que o subtipo receptor P2X3 é exclusivamente expresso em neurônios sensoriais de diâmetro pequeno (Namasivayam et al., 1999).

Purinoceptores P2X e P2Y medeiam a contração e relaxamento da musculatura lisa da bexiga urinária, respectivamente. A contração mediada por purinoceptores P2X nas células do músculo liso da bexiga urinária depende do influxo de íons de Ca^{2+} a partir do meio extracelular. Purinoceptores P2Y acoplados à proteína G, tais como $P2Y_1$, $P2Y_2$ e $P2Y_4$ são conhecidos pela

ativação de fosfolipase C e por aumentar a produção de inositol trifosfato (IP_3) (Naramatsu et al., 1997; Tempest et al., 2004; Rapp et al., 2005). Por outro lado, a ativação de receptores P2X leva ao rápido influxo de Na+ e Ca2+ e efluxo de K+, resultando na despolarização da membrana e um aumento de Ca2+ intracelular e ativação de quinases ativadas por mitógenos (MAP quinases) (Luttikhuizen et al., 2004).

Tempest e colaboradores (2004) mostraram pela primeira vez que o receptor P2X2 é expresso pelo urotélio humano, assim como os receptores P2X3 que já haviam sido anteriormente descritos por estarem expressos neste tecido. A função dos receptores P2X2 e P2X3 no urotélio da bexiga humana ainda não é completamente entendida, mas foi proposto que eles podem estar associados com a modulação da informação sensorial. Receptores $P2X_3$, que já são considerados como "receptores de dor" ao nível neuronal, podem também sugerir o envolvimento nos processos dolorosos da bexiga. Assim, a dor, o principal sintoma em cistite, poderia estar associada com as mudanças no sistema de sinalização purinérgica, como por exemplo, o aumento na liberação de ATP induzido pelo estiramento, bem como o aumento da expressão de receptores P2X2 e P2X3 (Tempest et al., 2004; Rapp et al., 2005; Dang et al., 2008).

Inicialmente, o receptor P2X3 foi clonado, sendo encontrado em altos níveis em neurônios sensoriais nociceptivos, propondo-se que este poderia ter um papel na dor aguda. Entretanto, estudos recentes sugerem que é muito mais provável que este receptor seja importante na mediação da dor crônica (Kennedy, 2005). Aplicado endogenamente, o ATP induz dor aguda em humanos e animais, a qual é inibida pela suramina, PPADS, e TNP-ATP; porém, endogenamente, o ATP não parece estar envolvido na nocicepção térmica ou mecânica, já que as respostas para estes estímulos apresentaram-se inalteradas em animais *knockout* para P2X3 (Cockayne et al., 2000; Souslova et al., 2000). Entretanto, estudos recentes têm demonstrado o papel dos receptores $P2X_3$ na dor associada com inflamação crônica e dano neural, sugerindo um papel central de desenvolvimento e manutenção de dor neuropática (Burnstock, 2006b). Outros dados revelaram que a artrite experimental é reduzida na ausência de receptores P2X7, mostrando a importância deste receptor como regulador da função inflamatória da célula e, sugerindo que níveis de ATP gerados endogenamente, como resultado da resposta inflamatória, são suficientes para ocupar este receptor ligado ao canal iônico (Labasi et al., 2002).

Os receptores P2X7 têm sido descritos em diversos sistemas, tais como cardiovascular, neurológico, sistema imune, gástrico, urogenital e respiratório, assim como em dermatologia e

oncologia (Burnstock, 2006b; Burnstock, 2007). Os receptores P2X7 existem em um número limitado de tipos celulares, mas são facilmente detectáveis em linhagens de células hematopoiéticas incluindo monócitos, macrófagos e linfócitos (Labasi et al., 2002; Chessell et al., 2005; Yoon et al., 2007; Chen et al., 2010). Dentro do sistema nervoso central, receptores P2X7 são localizados na microglia e células de Schwann, bem como em astrócitos (Donnelly-Roberts and Jarvis, 2007).

Os receptores P2X7 são ativados por altas concentrações de ATP e com maior potência pelo 2',3'-O-(4-benzoylbenzoyl)-ATP (BzATP); porém. o BzATP não é seletivo para o receptor $P2X_7$ (Donnelly-Roberts and Jarvis, 2007). A estrutura do receptor P2X consiste em três subunidades, sendo que cada uma delas é comparada a um golfinho saindo da superfície do oceano (membrana celular), com sua cauda submersa na bicamada lipídica, conforme apresentado na Figura 2b. Estes três "golfinhos" se entrelaçam mutuamente de forma a circunscrever o poro central vertical (Figura 2a). Na Figura 2c são apresentadas as pontes dissulfeto e, na Figura 2d é possível observar o local de ligação do ATP (Browne et al., 2010). Vários antagonistas dos receptores P2X7 têm sido identificados a fim de avaliar as funções deste receptor. Foi publicado um estudo com a caracterização completa de um antagonista do receptor P2X7, AZ11645373, que mostrou alta afinidade pelo receptor de humanos, porém apresentou baixa afinidade por receptores de ratos (Stokes et al., 2006). Outros antagonistas seletivos do receptor P2X7 e com afinidade comparável em humanos, ratos e camundongos tem sido desenvolvidos e caracterizados (Donnelly-Roberts and Jarvis, 2007; Donnelly-Roberts et al., 2009).

A ativação do receptor $P2X_7$ resulta em abertura rápida e reversível de canais que são permeáveis a Na+, K+ e Ca2+. O receptor serve também como um regulador da inflamação, pois está envolvido na produção de citocinas pró-inflamatórios, tais como TNFα e IL-1β, levando à indução de ciclooxigenase 2 (COX-2), metaloproteinases, óxido nítrico sintase (iNOS) e produção de superóxidos. Além disso, estão envolvidos na ativação de macrófago periférico e glial, infiltração de neutrófilos e produção de prostaglandinas (Labasi et al., 2002; Chessell et al., 2005; Chen and Brosnan, 2006; Di Virgilio, 2007b; Di Virgilio, 2007a; Donnelly-Roberts and Jarvis, 2007; King, 2007; Yoon et al., 2007; Chen et al., 2010; Burnstock, 2011).

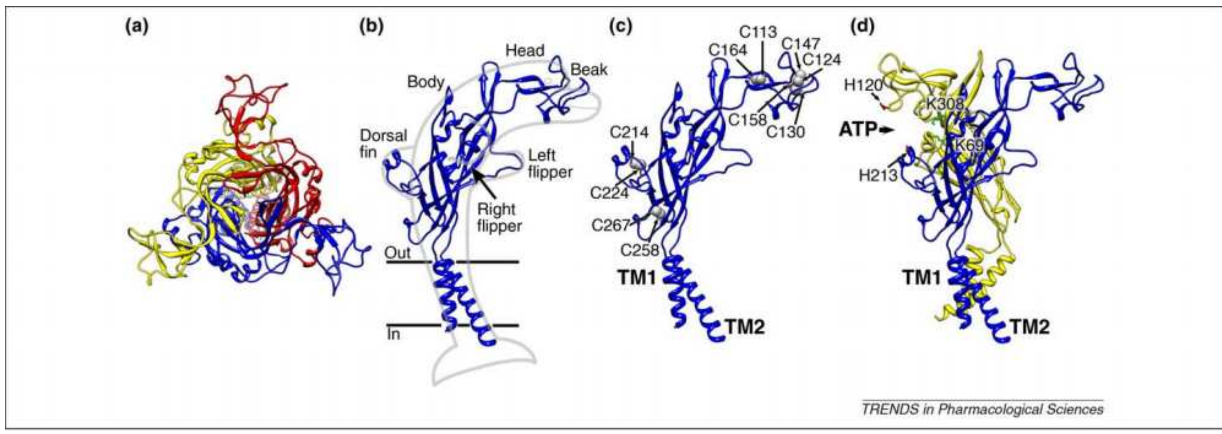

Figura 2. Estrutura do receptor P2X (Retirado de Browne et al., 2010; Trends in Pharmacological Sciences).

De maneira interessante, o receptor $P2X_7$ tem sido alvo de estudos para o desenvolvimento de novos analgésicos (Burnstock, 2006c; King, 2007; Burnstock, 2009; Honore et al., 2009; Burnstock, 2011). Assim, dados (Labasi et al., 2002) recentes obtidos com camundongos *knockout* para $P2X_7$ ou com novos antagonistas potentes e seletivos deste receptor indicam um papel crucial para o $P2X_7$ no início e na persistência de certos tipos de dor crônica (Donnelly-Roberts and Jarvis, 2007).

Gonçalves e colaboradores (2006) mostraram que os receptores $P2X_7$ estão envolvidos no processo de inflamação intersticial e deposição de colágeno, em um modelo de obstrução unilateral da uretra. Foi observado que camundongos *knockout* para o receptor $P2X_7$ apresentam menos macrófagos no interstício, menor população de miofibroblastos e deposição de colágeno, bem como, redução da expressão de TGF-β no interstício renal. Também foi demonstrado que a ausência deste receptor torna as células epiteliais menos apoptóticas, sugerindo que a ativação dos receptores $P2X_7$ poderia estar envolvida com a regulação da apoptose (Goncalves et al., 2006). Neste contexto, tem sido proposto que a exposição prolongada de receptores $P2X_7$ a agonistas leva à formação de um poro citolítico na membrana celular, permitindo assim a entrada de partículas maiores e conseqüente morte celular. (Burnstock, 2006b; Di Virgilio, 2007a; Donnelly-Roberts and Jarvis, 2007; Browne et al., 2010).

Considerando a gravidade dos quadros de cistite hemorrágica (CH) associados ao tratamento com os quimioterápicos CYP e ifosfamida e, ainda, o número limitado de opções terapêuticas para controlar as alterações inflamatórias e dolorosas relacionadas, torna-se imprescindível a realização de novos estudos, a fim de determinar os mecanismos responsáveis pelo estabelecimento deste processo patológico.

Nos últimos anos, tem aumentado o interesse no potencial terapêutico de componentes purinérgicos, incluindo agonistas e antagonistas de receptores, inibidores e estimuladores de ecto-enzimas e, inibidores e estimuladores de transporte de ATP (Kennedy, 2005; Burnstock, 2006b; Burnstock, 2006c; Burnstock, 2009; Burnstock, 2011). O ATP é gerado em resposta ao dano celular na inflamação e o receptor $P2X_7$ tem um papel essencial na inicialização ou manutenção de mudanças patológicas como um resultado de danos inflamatórios ou neuropáticos (Chessell et al., 2005; Di Virgilio, 2007a). De fato, a liberação de mediadores inflamatórios resultante da ativação do receptor P2X7, pode estar relacionada com as alterações inflamatórias e nociceptivas observadas na cistite hemorrágica (Wantuch et al., 2007). Neste contexto, o presente estudo teve por objetivo principal determinar o papel dos receptores P2X7 no modelo de cistite hemorrágica induzido pela aplicação de CYP em camundongos.

2. Objetivos

2.1. Objetivo Geral

O presente estudo teve por objetivo avaliar o papel dos receptores $P2X_7$ na cistite hemorrágica induzida por CYP em camundongos, através do uso de ferramentas farmacológicas, bem como de animais *knockout* para os receptores P2X7.

2.2. Objetivos Específicos

a. Avaliar os efeitos do bloqueio farmacológico ou deleção gênica dos receptores P2X7 nas alterações comportamentais induzidas pela CYP em camundongos;

b. Analisar os efeitos do bloqueio farmacológico ou deleção gênica dos receptores P2X7 sobre as alterações inflamatórias vesicais (edema e hemorragia), após a aplicação de CYP em camundongos;

c. Determinar os efeitos da inibição dos receptores P2X7 sobre a migração de células para a bexiga, induzida pela aplicação de CYP em camundongos, através de análise histológica;

d. Avaliar os efeitos do bloqueio farmacológico ou deleção gênica dos receptores P2X7 sobre a produção local de citocinas, causada pela CYP em camundongos;

e. Avaliar a expressão dos receptores P2X7 em animais tratados com CYP, em comparação a animais controle, através de análise imunoistoquímica;

f. Analisar a ativação do marcador de atividade neuronal, c-fos, em medulas e cérebros de camundongos tratados com CYP, bem como, investigar os efeitos do bloqueio farmacológico ou deleção gênica dos receptores P2X7 sobre este parâmetro.

3. Artigo Científico

Os resultados do presente trabalho foram submetidos ao periódico *British Journal of Pharmacology*

Fator de Impacto (ISI Web of Knowledge, 2009): 5,204

Submission Confirmation

Thank you for submitting your manuscript to *British Journal of Pharmacology*.

Manuscript ID:	2011-BJP-0069-RP
Title:	P2X7 purinergic receptor and its role in inflammatory and nociceptive alterations associated to cyclophosphamide-induced hemorrhagic cystitis in mice
Authors:	Martins, Jerônimo Silva, Rodrigo Coutinho-Silva, Robson Takiya, Christina Battastini, Ana Maria Morrone, Ferananda Campos, Maria
Date Submitted:	19-Jan-2011

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P2X7 purinergic receptor and its role in inflammatory and nociceptive alterations associated to cyclophosphamide-induced hemorrhagic cystitis in mice

[1]J.P. Martins, [2]R.B.M. Silva, [3]R. Coutinho-Silva, [4]C.M. Takiya, [5]A.M.O. Battastini, [2,6]F.B. Morrone, [6,7]M.M. Campos

[1]Faculdade de Medicina and [2]Farmácia, PUCRS, Porto Alegre, RS; [3]Instituto de Biofísica Carlos Chagas Filho, and [4]Instituto de Ciências Biomédicas, UFRJ, Rio de Janeiro, RJ; [5]Departmento de Bioquímica, ICBS, UFRGS, Porto Alegre, RS; [6]Instituto de Toxicologia, PUCRS , Porto Alegre, RS; [7]Faculdade de Odontologia, PUCRS, Porto Alegre, RS, Brazil.

Running title: P2X7 receptors and mouse hemorrhagic cystitis.

Corresponding Author: Maria Martha Campos, School of Dentistry/Institute of Toxicology, Pontifícia Universidade Católica do Rio Grande do Sul, Avenida Ipiranga, 6681, Partenon, 90619-900, Porto Alegre, RS, Brazil. Phone number: 55 51 3320 3562; Fax number: 5551 3320 3626.

E-mail: camposmmartha@yahoo.com; maria.campos@pucrs.br

Summary

Background and purpose: ATP is released in response to cellular damage, and P2X7 receptors have an essential role in the onset and maintenance of pathological changes. Haemorrhagic cystitis (HC) is a well known adverse effect of therapy with cyclophosphamide (CYP) used in patients under the treatment of many solid tumours and autoimmune conditions. This study aimed to determine the role of P2X7 receptors in the mouse model of HC induced by CYP.

Experimental approach: Effects of pharmacological antagonism or genic absence of P2X7 receptor in CYP-induced HC was assessed in a series of nociceptive and inflammatory parameters. In addition the immunopositivity for P2X7 receptors was investigated by means of immunohistochemistry.

Key results: The pre-treatment with the selective P2X7 receptor antagonist A438079 or genic ablation of P2X7 receptors inhibited the nociceptive behaviour score induced by CYP. The same strategies produced a significant reduction of both oedema and haemorrhage indexes, as indicated by either macroscopic or histological evaluation. A438079 treatment also significantly decreased the positive staining for c-Fos in the lumbar spinal cord and brain cortical areas. Noteworthy, the administration of A438079 markedly prevented the increase of urinary bladder MPO activity and macrophage migration induced by CYP, and widely reduced the tissue levels of IL-1β and TNFα. Finally, P2X7 receptor was found strikingly up-regulated in the bladders of CYP-treated mice.

Conclusions and implications: Our study revealed the importance of P2X7 receptors in HC induced by CYP. The pharmacological inhibition of these receptors might represent a new therapeutic alternative for this pathological condition.

Keywords: P2X7 receptor; haemorrhagic cystitis; cyclophosphamide; A438079; genic ablation, mice.

Abreviations

CYP, cyclophosphamide; HC, Haemorrhagic cystitis; Mesna, 2-mercaptoethane sulfonate; TBS, Tris-Buffer-Saline; BSA, bovine serum albumin; MPO, Myeloperoxidase; HTAB, hexadecyltrimethyl ammonium bromide; COX-2, cyclooxygenase-2; iNOS, inducible nitric oxide synthase; KO, Knockout; PBS, Phosphate Buffered Saline.

Introduction

Hemorrhagic cystitis (HC) is an inflammatory alteration of the urinary bladder, represented by hematuria, symptoms of local irritability (dysuria, frequency and urgency of urination) and pelvic pain. HC can be originated by radiation or by chemical agents, such as busulfan, oxazaphosphorines (cyclophosphamide and ifosfamide), and occasionally by penicillins and non-stereoidal anti-inflammatory drugs. Many cases of slight HC are spontaneously solved; nevertheless, in some cases, moderate to severe HC might result in significant morbidity, or even mortality (Traxer et al., 2001; Nickel, 2002; Neheman et al., 2005; Giraud et al., 2006; Cheuk et al., 2007; Manikandan et al., 2010).

The alkylating agent cyclophosphamide (CYP) acts in an unspecific manner on the cellular cycle, and it is commonly used for the treatment of many solid tumours, B cell malignancies, and as an immunosuppressive agent for autoimmune diseases, such as lupus and rheumatoid arthritis. It is a pro-drug, which is metabolized by hepatic microsomal enzymes, generating its active metabolite phosphoramide mustard, and the toxic agent acrolein. Acrolein is highly toxic to the urinary bladder, by promoting the release of inflammatory mediators, which ultimately lead to HC development (Crocitto et al., 1996; Wong et al., 2000; Traxer et al., 2001; Batista et al., 2006; Manikandan et al., 2010).

Patient hydration, bladder instillation and the use of diuretics lead to increased urinary flux, favouring the elimination of acrolein. In addition, some agents able to neutralize the toxic metabolite, such as sodium 2-mercaptoethane sulfonate (Mesna), have also been broadly used to prevent CYP-induced HC (Traxer et al., 2001). Nevertheless, once HC is installed, these methods have only minor beneficial effects. Some articles also describe the use of other strategies, such as antifibrinolytic drugs, potassium alum, silver nitrate, phenol, prostaglandins, formalin, arterial embolisation, hyperbaric oxygen therapy and others to treat HC, but all of them display limited

efficacy (Traxer et al., 2001; Neheman et al., 2005; Chow et al., 2006; Chow et al., 2007; Manikandan et al., 2010)).

The purinergic system is largely implicated in several of pathophysiological events. Purines exert their main effects by interacting with purinergic receptors, denoted P1 and P2 (Ralevic and Burnstock, 1998; Burnstock, 2011). P1 G protein-coupled receptors preferentially bind to adenosine, whereas P2 receptors recognize the nucleotides ADP, ATP, UTP and UDP. The P2 receptors are divided into two families: P2Y receptors (metabotropic) with eight subtypes ($P2Y_{1,4,6,11-14}$), and P2X receptors (ionotropic) with seven subtypes (P2X1-7) (Burnstock, 2007; Burnstock, 2009; Surprenant and North, 2009; Burnstock, 2011). Several P2 receptor subtypes are expressed in the urinary bladder and are likely involved in functions such as sensory and motor transmission under normal conditions. Understanding the involvement of these receptors in the pathophysiology of micturition, urinary dysfunction, and mainly in disease states might allow identifying new therapeutic targets to treat bladder maladies (Burnstock, 2009; Burnstock, 2011).

Amongst the P2X receptor family, the P2X7 subtype presents a series of peculiar features. Firstly, its activation requires near-millimolar concentrations of ATP (for up to 300 μM), whilst the other P2X receptors display a very high sensitivity for ATP. Next, P2X7 receptors are highly expressed in immune and inflammatory cells, throughout either the peripheral or the central nervous system. Latter, the activation of P2X7 receptors results in Ca^{+2} and Na^{+} influx and efflux of K^{+}, allied to the release of the pro-inflammatory cytokine interleukin-1β (Chen and Brosnan, 2006; Donnelly-Roberts and Jarvis, 2007; Dubyak, 2007; Skaper et al., 2010; Burnstock, 2011). Of high interest, this receptor has been implicated in peripheral macrophage and glia activation, neutrophil infiltration, and prostaglandin production (Labasi et al., 2002; Chessell et al., 2005; Burnstock, 2006; Di Virgilio, 2007; Donnelly-Roberts and Jarvis, 2007; King, 2007; Yoon et al.,

2007; Burnstock, 2011). Outstandingly, a series of recent studies have demonstrated the relevance of P2X7 receptors in several experimental models of disease, such as depression, epilepsy, Parkinson's disease, arthritis, cancer and chronic pain, by using either selective receptor antagonists or P2X7 receptor-null mice (Chessell et al., 2005; Honore et al., 2006; Li et al., 2006; Donnelly-Roberts and Jarvis, 2007; Basso et al., 2009; Marcellino et al., 2010; Teixeira et al., 2010).

Taking into account the abovementioned literature data, the present study was designed to evaluate whether P2X7 receptors are implicated in inflammatory and nociceptive alterations in a mouse model of HC induced by CYP administration. For that purpose, we have evaluated the effects of the selective P2X7 receptor antagonist A438079. When it was possible, we have also assessed the inflammatory and painful responses related to CYP-induced HC in mice with genic ablation of P2X7 receptors. To our knowledge, this is the first study aimed at investigating the relevance of purinergic P2X7 receptors in bladder inflammation.

Materials and Methods

Animals

Male Swiss, C57/BL6 and P2X7 receptor knockout (KO) mice (25 to 30 g) were used throughout this study. Swiss and C57/BL6 mice were obtained from Universidade Federal de Pelotas (UFPEL; Pelotas, RS, Brazil), and P2X7 receptor KO were donated by Dr. Robson Coutinho, Federal University of Rio de Janeiro (UFRJ, Rio de Janeiro, Brazil). The P2X7 receptor KO mice were generated by the method developed by Dr. James Mobley (PGRD, Pfizer Inc, Groton, CT, USA). The P2X7 receptor-deficient mice used in the present study were C57/BL6 inbred.

The animals were housed in groups of five per cage and maintained in controlled temperature (22±2ºC) and humidity (60-70%), under a 12 h light-dark cycle, with food and water *ad libitum*. All the experimental procedures were carried the out in accordance with the Guidelines for the Use and Care with Laboratorial Animals from National Institute of Health and ethical guidelines for investigations of experimental pain in conscious animal, and were approved by the Local Animal Ethics Committee (protocol number 08/00074). The number of animals and the intensity of noxious stimuli were the minimum necessary to demonstrate the consistent effects of the drug treatments.

Drugs and reagents

The following drugs were used: cyclophosphamide (Genuxa1@200® and Mesna (Mitexan®) are from Baxter Oncology Gmbh (Frankfurt, Germany), A438079 (3-((5-(2,3-dichlorophenyl)-1*H*-tetrazol-1-yl)methyl pyridine; Tocris, Bristol, UK). Hexadecyltrimethyl ammonium bromide and tetramethylbenzidine (Sigma-Aldrich, St. Louis, MO). $NaPO_4$, hydrogen

peroxide, NaCl and Tween 20 (all from Merck, Haar, Germany). All the dilutions were made in NaCl 0.9% (saline solution).

Treatments

HC was induced by a single administration of CYP (300 mg kg^{-1}) (Olivar and Laird, 1999; Wantuch et al., 2007). The treatment with the selective P2X7 receptor antagonist A438079 (100 and 200 μmol kg^{-1}) or with the reference compound Mesna (60 mg kg^{-1}) was performed in two doses; the first one was given 30 min prior CYP, and the following dose was administered 4 h after the injection of CYP, except in the experiments for assessing cytokines, in which the drugs were administered as a single i.p. dose, 30 min prior to CYP. The doses of A438079 or Mesna were chosen on the basis of the literature data (Batista et al., 2006; McGaraughty et al., 2007).

In separate groups of experiments, the relevance of P2X7 receptors was assessed by using animals with genic deletion for this receptor. C57/BL6 mice were used as the control group for this series of experiments. HC was induced as described beforehand.

Behavioural studies (Nociception)

The method used in the present study was similar to that described by Olivar & Laird (1999), with minor modifications. Experiments were performed between 8:00 and 12:00 AM to minimize the potential circadian variations in the behavioural responses. Immediately after the i.p. injection of CYP, mice were housed in individual plastic cages without sawdust bedding, and the spontaneous behaviour was measured for 2 min, every 30 min, in a total period of 4 h. The following behavioural changes were evaluated: (i) activity (walking, rearing, climbing, grooming etc.); (ii) immobility; and (iii) behaviours indicative of visceral pain (‘crises’). In addition, the

behavioural alterations were scored according to the following scale: 0 = normal; 1 = piloerection; 2 = strong piloerection; 3 = laboured breathing; 4 = licking of the abdomen; or 5 = stretching and contractions of the abdomen. At the end of the 4-h observation period, an open-field test was carried out. The animals were placed individually in a box divided in 9 squares, for 5 min. The time spent in the following behavioural categories was recorded: (i) rearing, (ii) walking, and (iii) not exploring (grooming, immobility). The number of squares crossed with the four paws was also registered, and taken as an index of locomotor activity (Olivar and Laird, 1999; Wantuch et al., 2007).

Gross evaluation

For this series of experiments, the animals were euthanized 6 h following CYP administration. The gross evaluation was based on criteria established by Gray et al. (1986). All bladders were dissected free from connecting tissues, and transected at the bladder neck. Each bladder was macroscopically evaluated, by an examiner blind to the experimental groups. The oedema formation was categorized as severe (3), moderate (2), mild (1) or absent (0). The oedema was considered severe when fluid was seen externally in the walls of the bladder, as well as internally. When the oedema was confined to the internal mucosa, it was reported as moderate; when it was between normal and moderate, the oedema was defined as mild. Upon examination, the bladders were also surveyed for bleeding in the walls and categorized into four designations, considering the presence of intravesical clots (3), mucosal hematomas (2), telangiectasia or dilatation of the bladder vessels (1), or normal aspect (0). Moreover the wet weight of each bladder was recorded, and expressed as milligram per 100 g of animal, as an additional measure of bladder oedema (Gray et al., 1986).

Histological analysis

Following the gross evaluation, the bladders were fixed in buffered formalin solution (10 %) for 24 h. After this period, the samples were embedded in paraffin, and stained with haematoxylin and eosin. A pathologist who was blinded to the treatment reviewed each specimen, considering the following parameters, as proposed by Gray et al. (1986), with some modifications: normal (normal epithelium, no inflammatory cell infiltrate or ulcers); mild (diminished epithelial cells, flattening with submucosal oedema, mild hemorrhage, few ulcerations); moderate (mucosal erosion, inflammatory cell infiltrate, fibrin deposition, haemorrhage, and multiple ulcerations); severe (mucosal erosion, inflammatory cell infiltrate, fibrin deposition, multiple ulcerations, and transmural haemorrhage with severe oedema).

Immunohistochemistry for c-Fos

The expression of c-Fos, a known biochemical marker of nociception, was measured by immunohistochemistry, as previously described by Labrousse et al. (2009). The lumbar spinal cords and the brains were rapidly excised (6 h after CYP application), and fixed in buffered neutral formalin. Sections were mounted onto gelatine-coated slides. Rabbit polyclonal antiserum raised against c-Fos (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA) was diluted in Tris-Buffer-Saline (TBS) containing 0.3 % Triton X-100, 2 % donkey serum and 1 % bovine serum albumin (BSA), and the sections were incubated overnight at room temperature, before being incubated for 2 h with biotinylated donkey anti-rabbit antibody (1:1000; Amersham Pharmacia Biotech Europe, Freiburg, Germany), for 2 h with avidin-biotin peroxidase complex (1:1000; Vectastain ABC kit, Vector laboratories, Burlingame, CA), and finally revealed with diaminobenzidine via the nickel-enhanced glucose-oxidase method. The procedure also included

negative controls with omission of the primary antibody, which did not show any immunoreaction. The images were captured by a digital camera (DS-5M-L1, Nikon, NY, USA), connected to an optical microscope (Nikon Eclipse 50i) and analyzed through the Image NIH Image J 1.36b Software. The number of c-Fos-positive cells was quantified and expressed as the positive area per filed (Labrousse et al., 2009).

Immunohistochemistry for F4/80 and P2X7 receptors

The bladder expression of P2X7 purinergic receptors and the macrophage migration were assessed by immunohistochemistry analysis. For these experiments, the bladders were collected 6 h after HC induction. Macrophages were quantified in the bladder tissues using the F4/80 rat anti-mouse macrophage monoclonal antibody (1:100; Serotec Ltd, Oxford, UK), while the expression of P2X7 receptor was determined by using the rabbit anti-P2X7 receptor-purified polyclonal antibody (1:200; Alomone, Jerusalem, Israel, catalog number APR-004). The bladders were rapidly excised 6 h after HC induction by CYP. For the immunostaining study, the general procedures described in the previous section were adopted.

Myeloperoxidase (MPO) activity

Neutrophil recruitment to the mouse bladder was measured by means of tissue MPO activity, according to the method described before (Passos et al., 2004). The bladders were removed at different time-points (1, 2, 4, 6, 8 and 12 h) after CYP injection. The tissues were homogenized at 5 % (wv^{-1}) in EDTA/NaCl buffer (pH 4.7) and centrifuged at 5,000 r.p.m. for 15 min, at 4°C. The pellet was resuspended in hexadecyltrimethyl ammonium bromide (HTAB) 0.5 % buffer (pH 5.4), and the samples were re-centrifuged (5,000 r.p.m., 15 min, 4°C). Twenty-five µl of the supernatant were used for the MPO assay. The enzymatic reaction was assessed with

tetramethylbenzidine 1.6 mM, NaPO4 80 mM and hydrogen peroxide 0.3 mM. The absorbance was measured at 595 nm, and the results are expressed in optical density per milligram of tissue. As the increase in MPO activity peaked at 6 h following CYP administration, this time interval was adopted for additional experiments on MPO activity determination.

Determination of cytokine production

The levels of IL-1β and TNF-α were measured according to the protocol described by Fernandes et al. (2005), with minor adaptations (Fernandes et al., 2005). The animals were treated with CYP (300 mg kg^{-1}, i.p.), and the bladders were collected at 4 h. Tissues were placed on PBS containing Tween-20 0.05 %, phenylmethylsulphonyl fluoride 0.1 mM, benzethonium chloride 0.1 mM, EDTA 10 mM and aprotinin A 20 KIU, homogenized, centrifuged at 3000 × g for 10min and stored at –70°C until further analysis. Cytokine levels were evaluated using specific ELISA kits, according to the manufacturer's recommendations (R&D Systems).

Statistical analysis

Results are expressed as the Mean ± Standard Error Mean. The percentages of inhibition were determined for each individual experiment. The statistical analysis was performed by one or two way analysis of variance (ANOVA), depending on the experimental protocol, followed by Bonferroni's post-hoc test. *P* values smaller than 0.05 were considered as significant. All tests were performed using the GraphPad® 4 Software (USA).

Results

Antagonism or absence of P2X7 purinergic receptor decrease nociceptive-related behaviour associated to HC

Confirming and extending previous literature data (Olivar and Laird, 1999), the i.p. injection of CYP (300 $mg.kg^{-1}$) was able to induce a marked and time-related increase in the behaviour nociceptive score, in either Swiss or C57/BL6 mice, a parameter that was visibly reduced by pre-treating animals with the reference compound Mesna (Figure 1).

Interestingly, the systemic administration of the selective P2X7 receptor antagonist A438079 (100 $\mu mol.kg^{-1}$; i.p.) resulted in a significant inhibition of the nociceptive behaviour evoked by CYP, as assessed in Swiss mice (43 ± 1 %). The same treatment with A438079, at the dose 200 $\mu mol.kg^{-1}$, also significantly reduced the nociceptive response (45 ± 9 %), an effect that was not significantly different from that observed for the dose of 100 $\mu mol.kg^{-1}$ (Figure 1B). Additionally, P2X7 receptor KO mice displayed a partial reduction of the nociceptive behavioural score (Figure 1D), with a mean inhibition percentage of 17 ± 3 %, when compared to C57/BL6 mice. Of note, either the pharmacological blocking or the genic ablation of P2X7 receptors produced significant reductions of CYP-induced nociceptive changes between 2.5 h and 4 h of evaluation (Figure 1A and C). Furthermore, none of the treatments (A438079 and Mesna), or genic deletion of P2X7 receptors, produced significant changes of rearing, walking, or general exploring, according to assessment in the open-field arena (results not shown).

Antagonism of P2X7 receptors is able to modulate the increased c-Fos expression in central structures of CYP-treated mice

As described above, the nociception associated to CYP-evoked HC was significantly inhibited by blocking P2X7 receptors. In an attempt to determine whether the effects of A438079

might involve the modulation of central pathways of pain transmission, we have tested the effects of this antagonist on c-Fos expression in the lumbar spinal cord and the brain cortical areas of mice treated with CYP.

The administration of CYP induced an expressive increase in the levels of c-Fos, in both the spinal cord and the brain, in comparison to the saline control groups. Interestingly, the i.p. treatment with A438079 (100 µmol.kg^{-1}) produced a striking decrease of c-Fos levels, in either anatomical structures (Figure 2A and B). The obtained percentages of inhibition were 86 ± 5 % and 45 ± 13 %, for the brain and the spinal cord, correspondingly. The treatment with Mesna (60 mg.kg^{-1}) also produced a reduction of c-Fos expression, in 73 ± 18 % and 71 ± 17 %, respectively (Figure 2A and B). Representative images of immunostaining for c-Fos are provided in the Figure 2C to J. In the lumbar spinal cords, the immunopositivity for c-Fos protein was found mainly in the lateral and the medial dorsal horn, as well as in the sacral parasympathetic nucleus (lanes G to J). Regarding the brain, the c-Fos immunopositivity was predominantly located in the cortical areas (lanes C to F); for this reason only this area has been considered for the analysis.

Blocking P2X7 purinergic receptors decreases oedema and haemorrhage caused by CYP

As described beforehand (Olivar and Laird, 1999; Santos et al., 2010), the damage scores of oedema and haemorrhage in the CYP group were significantly higher than in the saline groups. As well, the inflammatory changes associated to CYP-induced HC were similar in Swiss and C57/BL6 mice. According to the gross evaluation of the bladders, the positive control drug Mesna (60 mg.kg^{-1}) caused a significant inhibition of the haemorrhage (69 ± 12 % and 36 ± 1 %) and the oedema (80 ± 8 % and 84 ± 17 %) induced by CYP, in either the Swiss or the C57/BL6 mouse lineages, respectively (Figure 3A-F). Interestingly, the administration of the selective

P2X7 receptor antagonist A438079 (100 μmol.kg^{-1} and 200 μmol.kg^{-1}) was able to markedly inhibit the oedema formation following the application of CYP (29 ± 8 % and 21 ± 9 %, respectively) (Figure 3A). Otherwise, the haemorrhage scores were significantly reduced by A438079, at the dose of 100 μmol.kg^{-1} (36 ± 11 %), whereas the dose of 200 μmol.kg^{-1} did not display any significant alteration of this parameter (Figure 3B). The oedema in response to CYP administration was significantly reduced in P2X7 receptor KO mice, with a percentage of inhibition of 33 ± 14 % (Figure 3D). Nonetheless, although the haemorrhage score was visibly reduced in KO mice when compared to the C57/BL6 CYP-treated animals, this effect was not statistically significant (Figure 3E).

The oedema induced by CYP was also assessed by determining the mean empty bladder wet weight per 100 g of body weight. The treatment with A438079 (100 μmol.kg^{-1} and 200 μmol.kg^{-1}) produced a patent reduction of the increased wet bladder weights, with inhibition percentages of 34 ± 7 % and 33 ± 5 %, respectively (Figure 3C). Mice with genic deletion of P2X7 receptors showed an apparent reduction of the wet bladder weight, in relation to the C57/BL mouse strain, although this effect was not found significant (Figure 3F).

Histological changes following CYP administration and the effects of P2X7 receptor inhibition

A brief description of the main histological findings is presented below and representative images to each group are provided in the Figure 4. Overall, the treatment with A438079 and genic deletion of P2X7 receptors produced a partial reduction of HC inflammatory characteristics. Histologically, the bladder wall appeared thickened in CYP-treated mice compared with controls and was associated with partial loss of the urothelium. The remaining urothelial cells in CYP-treated mice were abnormally large, when compared with saline controls (Figure 4A and D). Furthermore, histological evaluation showed that submucosal layer was

enlarged and presented severe oedema, fibrosis, haemorrhage and inflammatory cell infiltrate in both Swiss and C57/BL6 mice (Figure 4B and E)). The animals pre-treated with Mesna (60 mg kg^{-1}) displayed characteristics near to the normality (results not shown). The animals treated with A438079 (Figure 4C), as well as the P2X7 KO mice (Figure 4F) presented moderate mucosal ulceration, and mild to moderate oedema and haemorrhage. The smooth muscle had normal appearance in all groups.

P2X7 receptors and CYP-induced macrophage migration

According to the immunohistochemical analysis of the marker F480, HC induced by CYP administration was accompanied by the recruitment of macrophages to the bladder tissue, in either Swiss or C57/BL6 mice, as shown in the Figure 5. CYP-induced macrophage migration was visibly reduced by pre-treating either mouse strains with the reference compound Mesna (60 $mg.kg^{-1}$) (Figure 5A and B). Notably, the immunopositivity for F480 was significantly diminished by pre-treating Swiss mice with the selective P2X7 receptor antagonist A438979, at the dose of 200 $\mu mol.kg^{-1}$, by 49 ± 18 %, whereas the dose of 100 $\mu mol.kg^{-1}$ failed to significantly alter this parameter (Figure 5A). In addition, CYP-induced macrophage migration to the bladder was virtually abolished in P2X7 receptor KO mice, although this effect was not found statistically significant (Figure 5B).

Representative images of data described above are given in the Figure 6, indicating a marked positive staining for F480 in the bladder submucosal layer of animals in the CYP groups (lanes B and E), in comparison to saline groups (lanes A and D). The immunostaining for F480 was visibly diminished in either A438979-treated animals (lane C) or in P2X7 receptor KO mice (lane F).

P2X7 purinergic receptor immunostaining is increased following CYP treatment

The expression of P2X7 purinergic receptor in the normal human bladder has been previously described (O'Reilly et al., 2001b). Herein, we demonstrate that a single i.p. dose of CYP (300 mg.kg^{-1}) resulted in a significant increase of P2X7 receptor expression in the Swiss mouse bladder, with an increase of 229 ± 48 % in the immunopositivity, in relation to control saline-treated mice (Figure 7A). Representative images demonstrate low immunostaining for the P2X7 receptor in saline-treated animals (Figure 7B), whereas the immunopositivity for this receptor was markedly enhanced in the bladder submucosal layer of CYP-treated mice (Figure 7C).

Inhibition of MPO activity by P2X7 antagonist

CYP administration was also associated to a massive migration of neutrophils, as indicated by the increase of MPO activity in the bladder tissue, when compared to the saline group. The increase of MPO activity induced by CYP was similarly reduced by the treatment with Mesna (60 mg.kg^{-1}) and A438079 (100 μmol.kg^{-1}) (Figure 8A). The percentages of inhibition were 28 ± 6 % and 27 ± 8%, respectively. No significant differences were observed between treatments with Mesna and A438079.

Evaluation of cytokine levels in the bladder

TNF-α and IL-1β production was assessed in the bladder tissue 4 h after CYP administration. The results demonstrate that CYP induced a marked increase of both IL-1β (Figure 8B) and TNF-α (Figure 8C) levels. Interestingly, the treatment with Mesna (60 mg.kg^{-1}) or A430879 (100 μmol.kg^{-1}) was able to significantly reverse the increased production of IL-1β

and TNF-α to the basal levels (Figure 8). In the Mesna-treated groups, the reduction percentages were 40 ± 8 % and 77 ± 6 %, for IL-1β and TNF-α, respectively. For the groups treated with the selective P2X7 receptor antagonist A438079, the percentages of inhibition were 48 ± 8 % and 73 ± 7 %, for IL-1β and TNF-α, correspondingly. Again, no significant differences were observed between treatments with Mesna and A438079.

Discussion

The toxic metabolite of CYP named acrolein induces HC by extended contact with the bladder epithelium, causing cellular damage by promoting the release of several inflammatory mediators, including purine nucleotides. In fact, the increased release of ATP from the bladder urothelial cells has been associated with interstitial cystitis, and also occurs in response to bladder stretch or distension (Namasivayam et al., 1999; Sun and Chai, 2006). It has been demonstrated that P2X7 purinergic receptors, which are known to be activated by elevated ATP concentrations, are constitutively expressed in the bladder tissue under normal conditions (O'Reilly et al., 2001a; O'Reilly et al., 2001b; Birder et al., 2004). Of note, the same authors have shown that P2X7 receptors are found up-regulated in the urinary bladder of patients with symptomatic outlet obstruction (O'Reilly et al., 2001a; O'Reilly et al., 2001b). However, further studies are still required in order to determine whether these receptors might be relevant under other bladder pathological states, such as CYP-induced HC.

The present study brings new evidence on the pathological role of P2X7 receptors, by demonstrating, for the first time, that either the pharmacological blocking or the genic ablation of this receptor subtype is able to prevent either the nociceptive or the inflammatory events associated to HC evoked by CYP treatment in mice. Furthermore, our data revealed that CYP-induced HC was likely accompanied by increased expression of P2X7 receptors, supporting the relevance of this receptor in HC.

HC induced by CYP represents a useful experimental model to evaluate visceral pain in both rats and mice (Gray et al., 1986; Olivar and Laird, 1999; Bon et al., 2003; Batista et al., 2006; Wantuch et al., 2007). Pain is the principal symptom in cystitis, and might be associated with purinergic signalling changes, e.g. stretching-induced ATP release, as well as the up-regulation of purinergic receptors (Tempest et al., 2004). Relevantly, the P2X7 receptor subtype

has been investigated as a target to development of new analgesic drugs. On this regard, it has been recently demonstrated that P2X7 receptors display a pivotal role in the onset and persistence of certain types of inflammatory and neuropathic pain in rodents (Honore et al., 2006; Donnelly-Roberts and Jarvis, 2007; King, 2007; McGaraughty et al., 2007). Our results revealed that systemic treatment of mice with the selective P2X7 receptor antagonist A438079 markedly prevented the nociceptive changes related to CYP-evoked HC, in a grade comparable to the reference compound Mesna. Furthermore, the nociceptive behavioural changes elicited by CYP administration were also found diminished in P2X7 receptor null mice. Of high interest, none of tested strategies, the pharmacological antagonism or the genic deletion of P2X7, was able to significantly affect the general locomotor activity of mice in the open-field test, discarding possible unspecific central effects of P2X7 receptor inhibition. A similar result was previously demonstrated before, showing that P2X7 KO mice displayed antidepressant behaviour in the tail suspension and forced swimming tests, without changing in the spontaneous locomotor activity (Basso et al., 2009). Therefore, it is feasible to suggest that P2X7 receptors play a relevant role in HC-related painful alterations, and selective antagonists for this receptor might be attractive alternatives for treating clinical pain under cystitis.

As discussed beforehand, P2X7 receptors are physiologically expressed in the urinary bladder (O'Reilly et al., 2001a; O'Reilly et al., 2001b; Birder et al., 2004). Consequently, the analgesic effects resultant from the receptor inhibition might rely on the modulation of P2X7 receptors expressed in the bladder tissue or at peripheral sensory endings innervating this organ. Otherwise, the effects of P2X7 receptors could involve the modulation of central pathways of pain transmission, since these receptors are found highly expressed in the spinal cord (Cotrina and Nedergaard, 2009). Hence, we have tested the effects of A438079 on c-Fos expression in the lumbar spinal cord and the brain cortical areas following the administration of CYP to mice.

Immunohistochemical analysis demonstrated that c-Fos staining was markedly increased in either the lumbar spinal cord or the brain cortex of mice that received CYP. Strikingly, the immunolabelling for c-Fos was greatly inhibited by treating animals with A438079 in both anatomical structures, indicating that systemic administration of this antagonist is able to modulate pain processing at central levels. A series of previous studies have used the expression of the immediate early gene c-Fos as a marker for postsynaptic activation of spinal cord neurons receiving afferent inputs from the bladder. It has been shown that the number of c-Fos immunoreactive neurons increase following the injury of the lower urinary tract (Morais et al., 1999; Vizzard, 2000b; Vizzard, 2000a; Nickel, 2002). Data obtained on c-Fos immunostaining reinforces the notion pointing out P2X7 receptor antagonists as potential tools for treating pain related to cystitis.

Tissue bladder injury following chemotherapy with CYP is widely related to exacerbated inflammatory alterations induced by acrolein contact (Namasivayam et al., 1999; Sun and Chai, 2006). In the present study, we have also assessed to what extent the interference with P2X7 receptors might affect the inflammatory changes evoked by CYP administration. Confirming previous literature data (Olivar and Laird, 1999; Santos et al., 2010), the single treatment with CYP resulted in expressive oedema and haemorrhage formation, according to either gross or histological evaluation. Notably, the grade of oedema and bleeding was markedly reduced in mice pre-treated with the selective P2X7 receptor antagonist A438079, or even in animals with genic P2X7 receptor deletion, in an extent comparable to the clinically used strategy Mesna. We might conclude that either the nociceptive and inflammatory changes associated to CYP-induced HC involve the activation of P2X7 receptors. Additionally, we can also hypothesize that analgesic effects observed for A438079 are mediated, at least partially, by the modulation of the inflammatory responses. Indeed, P2X7 receptors are well recognized regulators of inflammation,

as they are involved in the production of pro-inflammatory cytokines, such as IL-1β, leading to the up-regulation of cyclooxygenase-2 (COX-2), metalloproteinases, inducible nitric oxide synthase (iNOS) and superoxide production (Labasi et al., 2002; Chessell et al., 2005; Di Virgilio, 2007; Donnelly-Roberts and Jarvis, 2007; Basso et al., 2009; Grassi, 2010). Furthermore, supporting our data, a recent study conducted by Riteau et al. (2010) demonstrated that P2X7 receptor null mice displayed a marked reduction of lung inflammation in a murine model of lung fibrosis.

The P2X7 receptor subtype has been implicated in peripheral macrophage and glial activation, as well as neutrophil infiltration (Labasi et al., 2002; Chessell et al., 2005; Burnstock, 2006; Di Virgilio, 2007; Donnelly-Roberts and Jarvis, 2007; King, 2007; Yoon et al., 2007; Burnstock, 2011).One might wonder whether the inhibition of P2X7 receptors would modulate inflammatory cells under CYP-induced HC. The results presented herein clearly demonstrate that CYP administration resulted in a marked increase of macrophage infiltration to the urinary bladder, according to the immunohistochemical evaluation of F4/80 staining. Interestingly, this parameter was markedly reduced by A438079 pre-treatment or in P2X7 receptor null mice. Allied to our data, Gonçalves and colleagues (2006) demonstrated that P2X7 receptor KO mice presented a notable reduction of interstitial macrophage migration, in an experimental model of unilateral urethral obstruction. Thus, it is possible to suggest that P2X7 receptors are likely implicated in the mechanisms of macrophage influx and/or proliferation under inflammatory diseases of the urinary tract.

MPO is a naturally-occurring enzyme contained within the primary granules of neutrophils, and the augmented activity of this enzyme represents an indirect measure of neutrophil migration (Passos et al., 2004). It has been previously shown that purinergic signalling is essential for neutrophil migration (Chen et al., 2010). Our work extends this evidence by

demonstrating that increased MPO activity following CYP administration was markedly prevented by pre-treating animals with the selective P2X7 receptor antagonist A438079. This assembly of results are clearly indicative that both macrophage and neutrophil migration in our experimental model of bladder inflammation is broadly dependent on the activation of P2X7 receptors.

It is well known that P2X7 receptors are responsible for modulating the maturation and the release of IL-1β in peripheral macrophages and microglia, via pannexin-1 hemichannels (Labasi et al., 2002; Chessell et al., 2005; Di Virgilio, 2007; Donnelly-Roberts and Jarvis, 2007; Iglesias et al., 2008; Basso et al., 2009; Grassi, 2010). An *in vitro* study conducted by Basso and co-workers (2009) showed that stimulation of P2X7 receptors by the selective agonist BzATP resulted in a marked production of IL-1β in macrophages obtained from wild-type, but not from P2X7 receptor KO mice. Additionally, a recent study conducted by Teixeira et al. (2010) indicated A438079 was able to significantly decrease carrageenan-induced mechanical hyperalgesia in rats, by interfering with the release of IL-6, TNFα and CINC-1, although the production of IL-1β was not affected. In the experimental model of HC elicited by CYP, the treatment of mice with the selective P2X7 receptor A438079 virtually brought the production of the pro-inflammatory cytokines IL-1β and TNFα to the basal levels. It is tempting to surmise that A438079 is able to interfere with cytokine production by indirectly modulating leukocyte migration/activation, or even by blocking the cytokine release from endothelial or bladder smooth muscle cells. Furthermore, we might also infer that part of the antinociceptive actions observed for A438079 are likely mediated by an interference with IL-1β and TNFα generation, as both cytokines are greatly implicated in sensitization of primary afferent nociceptors. Supporting this idea, a study performed by Honore et al. (2009) demonstrated that antinociceptive effects

displayed by the selective P2X7 receptor antagonist A839977 was found diminished in IL-1αβ KO mice, according to assessment in a chronic model of inflammation (Honore et al., 2009).

Present data obtained employing either the pharmacological antagonism or the genetic deletion of P2X7 receptors is actually conclusive on the relevance of these receptors in CYP-induced cystitis. Noteworthy, the antagonist used in our study has been demonstrated to be highly potent and selective in blocking the activation of human, rat and mouse P2X7 receptors (Donnelly-Roberts et al., 2009). Additionally, although genetically modified animals might exhibit compensatory changes in signalling transduction, KO mice (including the P2X7 one) generally represent a good tool to confirm functional data using pharmacological approaches (Chessell et al., 2005). To gain further insight on the relevance of P2X7 receptors in our experimental model, we have also investigated whether the single administration of CYP would be able to modulate the expression of P2X7 receptors in the bladder tissue. Immunohistochemical analysis provides compelling evidence on the up-regulation of purinergic P2X7 receptors in the urinary bladder of mice with CYP-induced HC, as demonstrated by an increase of about 3-fold in positive immunolabelling for this receptor. Relevantly, in our model, the immunopositivity for P2X7 receptors was mainly observed in the submucosal layer of bladders, allowing suggesting that receptor up-regulation probably takes place in immunological cells, for instance macrophages and neutrophils. An increased neuronal expression of P2X7 receptors was recently described in an experimental model of multiple sclerosis, at the peak of neurological symptoms, what is in accord with our results (Grygorowicz et al., 2010).

Taken together the results of the present study extend previous literature data, showing for the first time that P2X7 purinergic receptors are implicated in the inflammatory process of CYP-induced HC, probably by increasing macrophage and neutrophil migration, and generating pro-

inflammatory cytokines, which in turn might directly activate peripheral and central nociceptive pathways, or just facilitate the nociception processing. One could assume that P2X7 receptors would represent attractive targets for treating the symptoms related to severe HC in patients under chemotherapy with CYP.

Acknowledgements

This work was supported by grants from the Fundação de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERJ), and the Programa de Apoio aos Núcleos de Excelência (PRONEX), Brazil. JPM is a post-graduate student in Medicine and Health Sciences (PUCRS), supported by the Institution (*PROBOLSAS* Program/PUCRS). RBMS is an undergraduate Pharmacy student holding a fellowship from Conselho Nacional de Desenvolvimento Científico e Tecnológico (PIBIC, CNPq, Brazil).

Conflict of interest

The authors declare no conflict of interest.

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Figures:

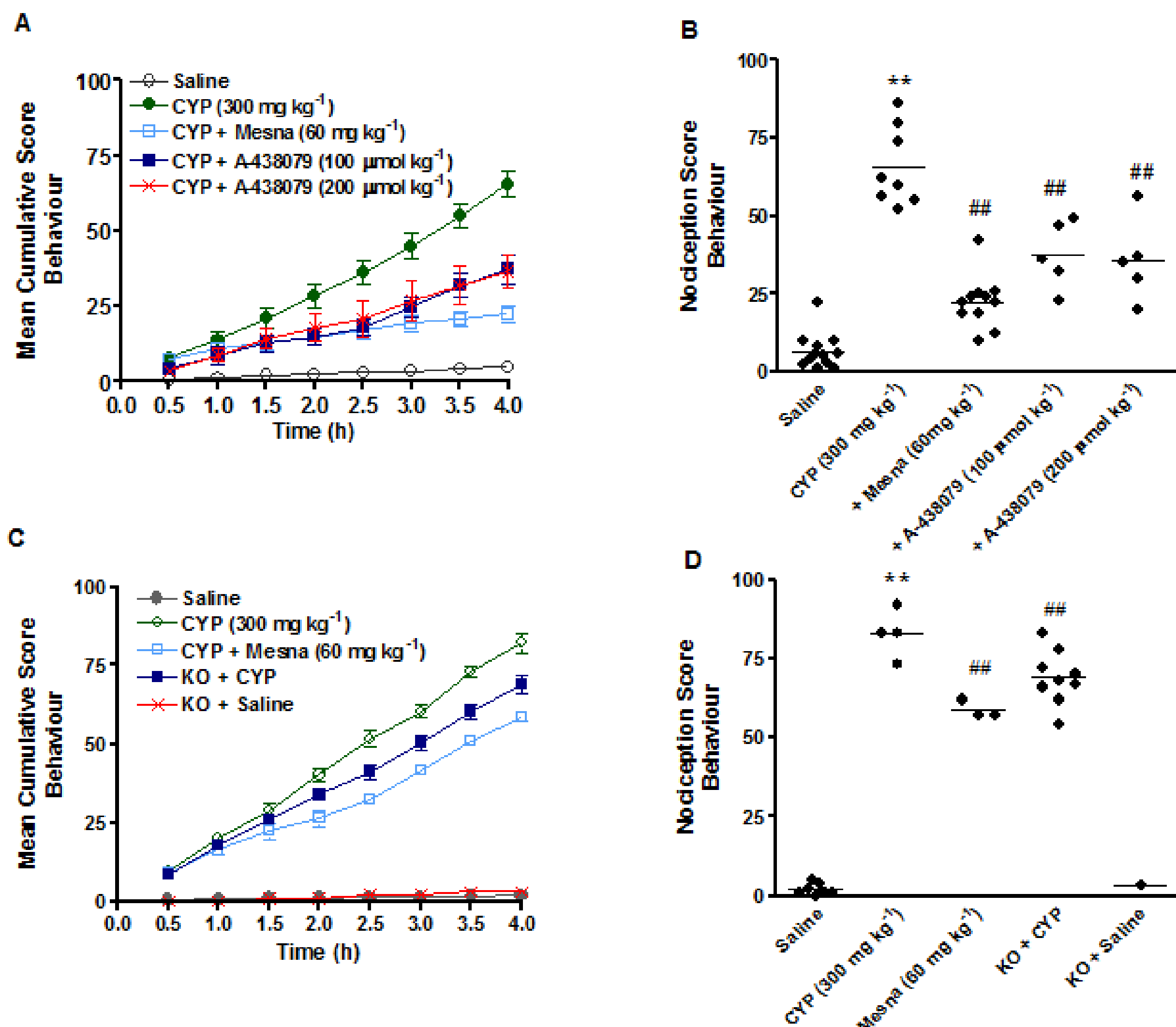

Figure 1 Behavioural scores assigned at 30 min intervals during 4-h post injection period. (A) Time-course of behavioural scores plotted as cumulative score over the 4-h observation period. (B) Effect of treatment with Mesna (60 mg kg^{-1}, i.p.) and A-438079 (100 or 200 µmol kg^{-1}, i.p.) on the nociceptive responses in CYP-induced HC in Swiss mice. (C) Time-course of behavioural scores plotted as cumulative score over the 4-h observation period. (D) Effect of treatment with Mesna (60 mg kg^{-1}, i.p.) or effect of P2X7 receptor genic deletion on the nociceptive responses in CYP-induced HC in C57/BL6 mice. Each column represents the mean of 3 to 10 animals, and the vertical lines show the S.E.M. **P<0.01 denotes the significance levels in comparison to saline values; ##P<0.01 denotes the significance levels in comparison to control (CYP) values.

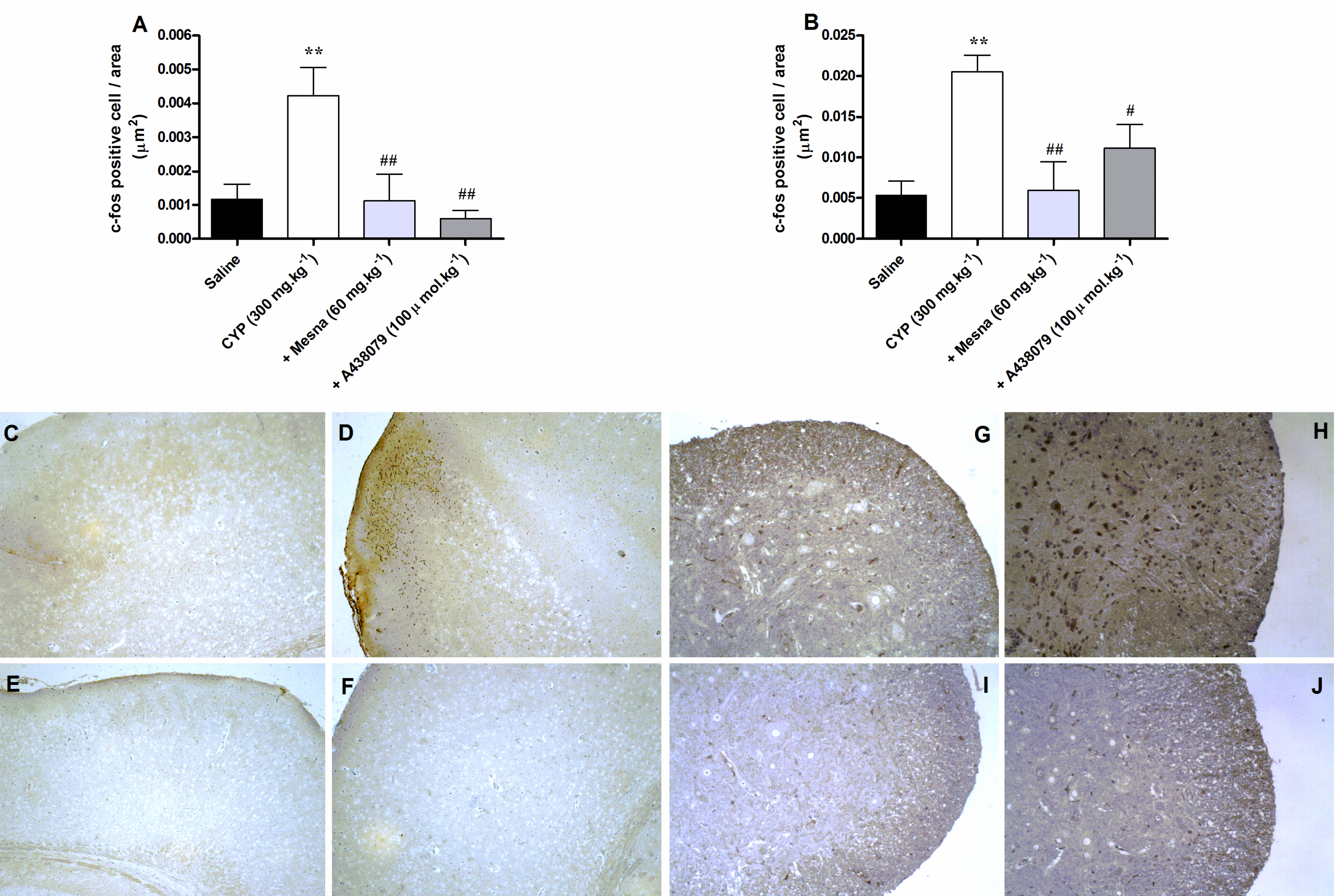

Figure 2 Effect of treatment with Mesna (60 mg kg^{-1}, i.p.) and A-438079 (100 µmol kg^{-1}, i.p.) on c-Fos expression in the brain cortical areas (A) and the lumbar spinal cord (B) in the CYP-induced HC model. (C to F) Representative images of immunostaining for c-Fos in the brain cortical areas into the following groups: (C) saline, (D) CYP, (E) CYP plus Mesna (60 mg kg^{-1}) and (F) CYP plus A-438079 (100 µmol kg^{-1}). (G to J) Representative images of immunostaining for c-Fos in the lumbar spinal cord into the following groups: (G) saline, (H) CYP, (I) CYP plus Mesna (60 mg kg^{-1}) and (J) CYP plus A-438079 (100 µmol kg^{-1}). Each column represents the mean of 5 to 8 animals, and the vertical lines show the S.E.M. **P<0.01 denotes the significance levels in comparison to saline values; #P<0.05 and ##P<0.01 denote the significance levels in comparison to control (CYP) values.

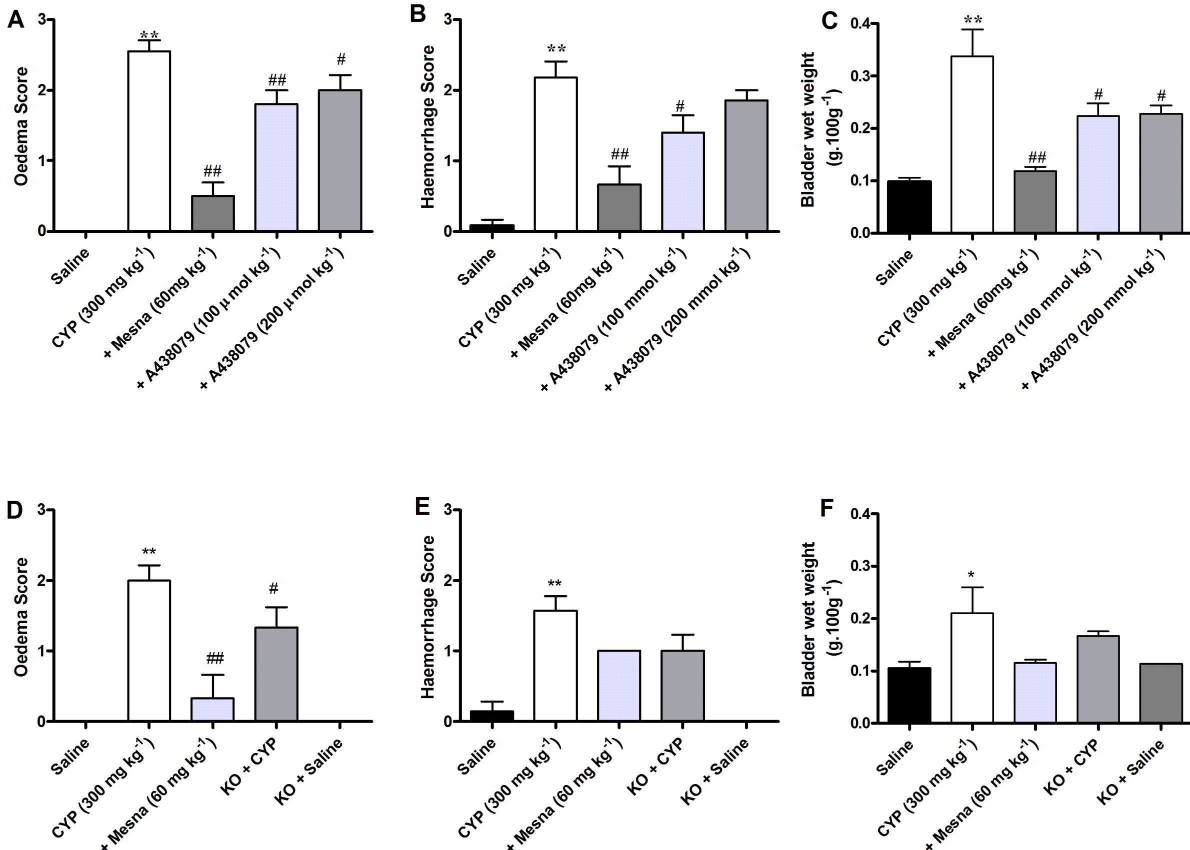

Figure 3 Effect of treatment with Mesna (60 mg kg^{-1}, i.p.) and A-438079 (100 or 200 µmol kg^{-1}, i.p.) on macroscopic oedema (A) and haemorrhage (B) evaluation, and on wet weight bladder (C) in CYP- induced HC in Swiss mice. Effect of treatment with Mesna (60 mg kg^{-1}, i.p.) or use of knockout P2X7 receptor mice on macroscopic oedema (D) and haemorrhage (E) evaluation, and on wet weight bladder (F) in CYP- induced HC in C57/BL6 mice. Each column represents the mean of 3 to 10 animals, and the vertical lines show the S.E.M. *P<0.05 and **P<0.01 denote the significance levels in comparison to saline values; #P<0.05 and ##P<0.01 denote the significance levels in comparison to control (CYP) values.

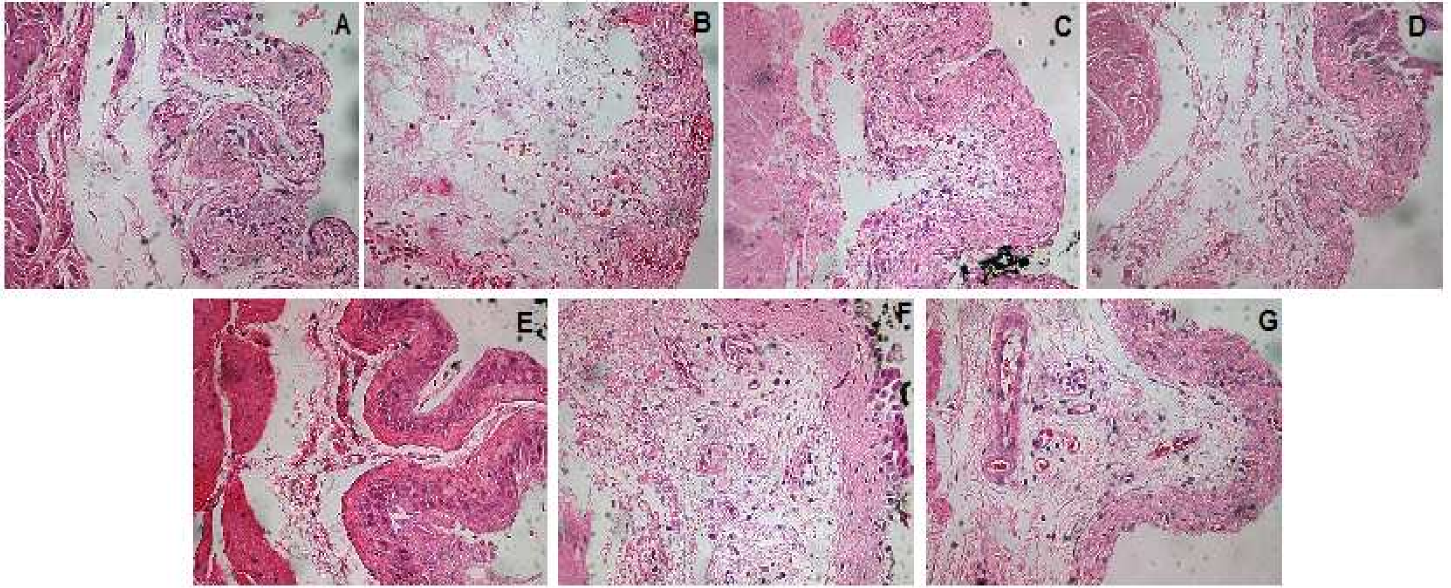

Figure 4 Representative images of histological evaluation in slides stained with with haematoxylin and eosin. Saline groups in (A) Swiss and (E) C57/BL6 mouse lineages; CYP groups in (B) Swiss and (F) C57/BL6 mouse lineages; animals treated with A-438079 (C), Mesna (D) or P2X7 receptor KO mice (G).

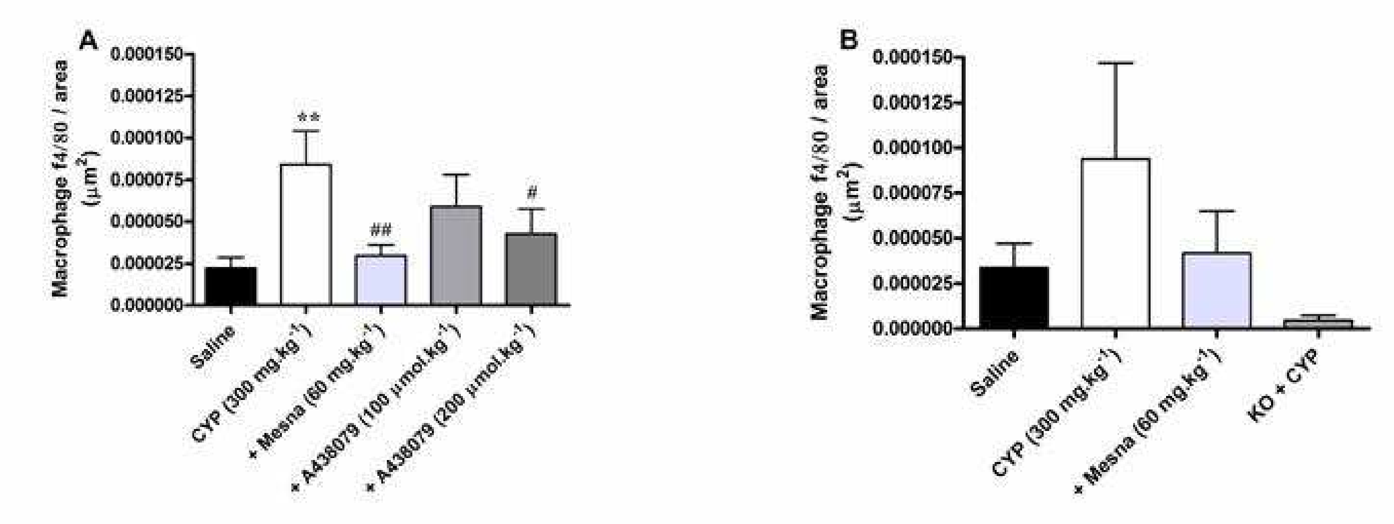

Figure 5 (A) Effect of treatment with Mesna (60 mg kg^{-1}, i.p.) and A-438079 (100 or 200 μmol kg^{-1}, i.p.) on bladder macrophage migration, as determined by immunohistochemistry, in the CYP-induced HC model. (B) Effect of treatment with Mesna (60 mg kg^{-1}, i.p.) or use of knockout P2X7 receptor mice on macrophage migration by immunohistochemical on bladder macrophage migration, as determined by immunohistochemistry, in the CYP-induced HC model. Each column represents the mean of 3 to 10 animals, and the vertical lines show the S.E.M. $^{**}P<0.01$ denotes the significance levels in comparison to saline values; $\#P<0.05$ and $\#\#P<0.01$ denote the significance levels in comparison to control (CYP) values.

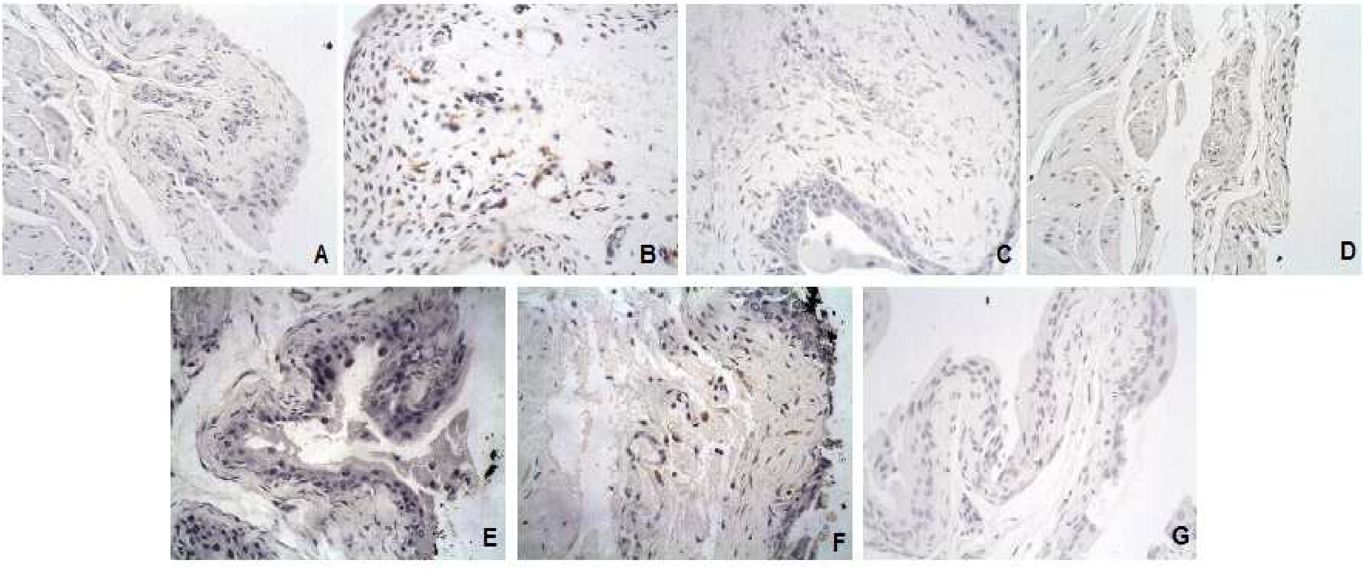

Figure 6 Representative images of immunostaining for F4/80 in the bladder submucosal layer. Saline groups in (A) Swiss and (E) C57/BL6 mouse lineages; CYP groups in (B) Swiss and (F) C57/BL6 mouse lineages indicating a marked positive staining for F4/80; animals treated with A-438079 (C), Mesna (D) or P2X7 receptor KO mice (G) displaying diminished immunostaining for F4/80.

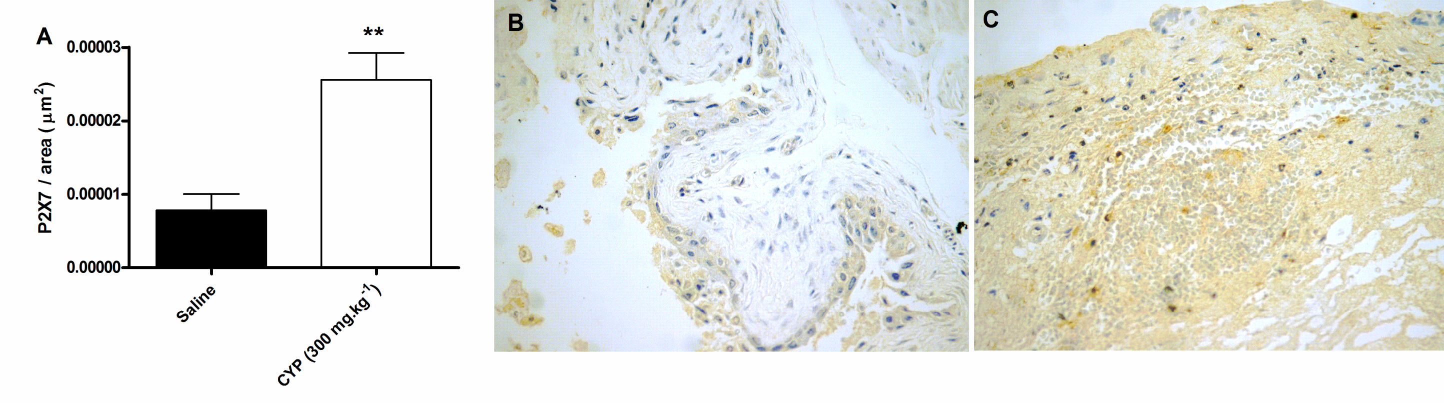

Figure 7 (A) Effect of treatment with CYP (300 mg kg^{-1}, i.p.) on P2X7 receptor expression in the mouse bladder submucosal layer of Swiss mice. Representative images demonstrate low immunostaining for the P2X7 receptor in saline-treated animals (B), whereas the immunopositivity for this receptor was markedly enhanced in the bladder submucosal layer of CYP-treated mice (C). Each column represents the mean of 6 animals, and the vertical lines show the S.E.M. **$P<0.01$ denotes the significance levels in comparison to saline values.

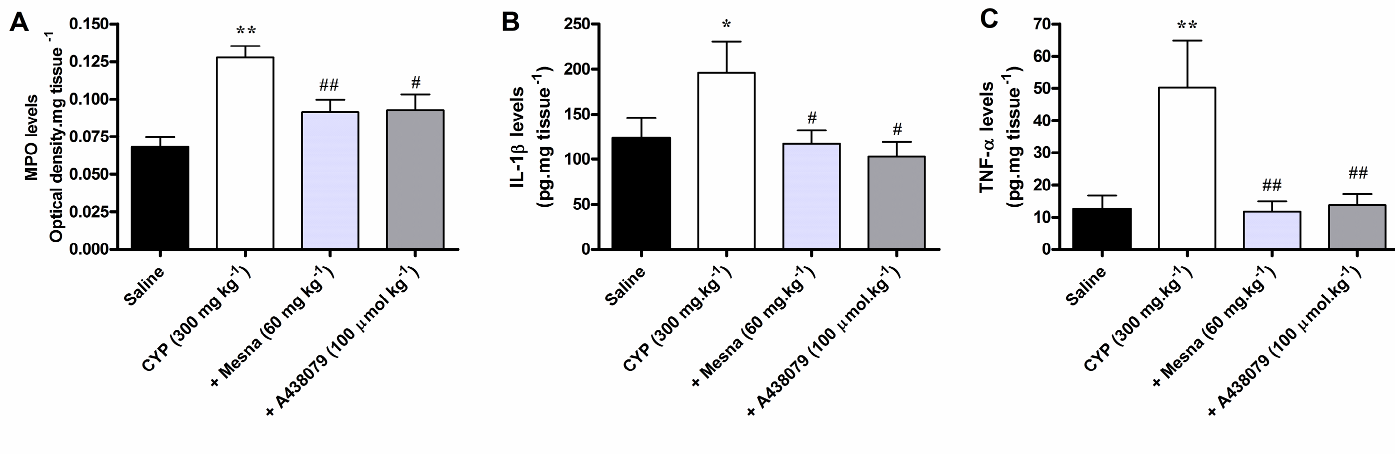

Figure 8 Effect of treatment with Mesna (60 mg kg^{-1}, i.p.) and A-438079 (100 μmol kg^{-1}, i.p.) on increased MPO activity (A) and on generation of IL-1β (B) and TNF-α (C) in CYP-induced HC in Swiss mice. Each column represents the mean of 6 animals, and the vertical lines show the S.E.M. *P<0.05 and **P<0.01 denote the significance levels in comparison to saline values; #P<0.05 and ##P<0.01 denote the significance levels in comparison to control (CYP) values.

4. Considerações Finais

Os receptores purinérgicos têm sido largamente estudados como alvo para o tratamento de doenças inflamatórias e dolorosas. Nos últimos anos, o número de artigos publicados relacionados ao receptor P2X7 aumentou consideravelmente devido ao seu envolvimento com a inflamação e outras patologias. Uma série de estudos recentes tem demonstrado a relevância do receptor P2X7 em modelos experimentais tais como depressão, epilepsia, doença de Parkinson, artrite, câncer e dor crônica, onde foram utilizados antagonistas seletivos ou animais *knockout* para este receptor (Chessell et al., 2005; Honore et al., 2006; Li et al., 2006; Donnelly-Roberts and Jarvis, 2007; Basso et al., 2009; Marcellino et al., 2010; Teixeira et al., 2010).

A sinalização purinérgica no controle da função do trato urinário tem sido estudada desde 1972. Os trabalhos mais recentes mostram o potencial terapêutico de compostos purinérgicos na incontinência urinária, dor e câncer (Burnstock, 2011). Na cistite intersticial, sabe-se que ocorre aumento dos receptores P2X2 e P2X3, estando associados com a modulação sensorial. Já, os receptores P2X7 apresentaram um aumento da expressão em pacientes com obstrução sintomática do canal uretral (O'Reilly et al., 2001a; O'Reilly et al., 2001b; Birder et al., 2004; Tempest et al., 2004; Rapp et al., 2005; Dang et al., 2008; Burnstock, 2011).

A CH associada ao uso dos medicamentos quimioterápicos, CYP e ifosfamida, é causada devido ao contato prolongado da acroleína (metabólito tóxico) com o urotélio da bexiga, que resulta na liberação de mediadores inflamatórios tais como TNF-α, IL-1β e óxido nítrico endógeno, causando edema na mucosa da bexiga, dilatação vascular e aumento da fragilidade capilar resultando em hemorragia. Uma vez que a via de maturação da caspase-1/IL-1β é modulada pelo receptor P2X7, é possível propor que este receptor possa estar envolvido no processo inflamatório relacionado à CH.

A Mesna tem sido vastamente utilizada na clínica em associação à CYP e ifosfamida, como uma forma de prevenir a CH. Porém, estudos recentes têm demonstrado que este medicamento apresenta efeitos adversos como dores de cabeça, diarréia e reações de hipersensibilidade, especialmente em crianças. Além disso, foi verificado que a Mesna e a hiper-hidratação são igualmente eficazes na prevenção da CH associada à CYP, com valores de 33 e 20%, respectivamente (Shepherd et al., 1991). Dessa forma, tem-se observado que as medidas de

prevenção da CH não evitam completamente a ocorrência da lesão e, após o estabelecimento da mesma, o número de alternativas terapêuticas é limitado.

Com o objetivo de encontrar alternativas de tratamento para a CH e avaliar a participação do receptor P2X7 nesta alteração patológica, o presente estudo investigou a participação destes receptores nas respostas inflamatórias e nociceptivas observadas no modelo de CH induzida pela CYP, *in vivo*, através do emprego de técnicas farmacológicas e do uso de animais geneticamente modificados. Os nossos resultados demonstraram que o bloqueio ou ausência do receptor P2X7 diminuem a nocicepção gerada na CH, através da avaliação de parâmetros comportamentais. As mesmas estratégias foram capazes de reduzir a expressão da proteína c-Fos, tanto na medula espinhal quanto no cérebro, indicando uma redução na transmissão da dor em níveis centrais. Os resultados sugerem que este receptor tem um papel relevante nas alterações dolorosas relacionadas à CH e parece estar envolvido na modulação das vias centrais da transmissão da dor. O uso de antagonistas seletivos para este receptor pode ser uma alternativa atrativa para tratar a dor relacionada à cistite, um dos principais sintomas deste quadro, que limitam em muito a qualidade de vida dos pacientes.

Outros resultados obtidos no presente estudo mostram que o antagonismo ou a ausência do receptor P2X7 foram capazes de reduzir a inflamação gerada pela acroleína na CH. Houve redução nos parâmetros macroscópicos (edema e hemorragia), histológicos e bioquímicos (geração de TNF-α e IL-1β, migração de macrófagos e neutrófilos), indicando que o receptor P2X7 está envolvido na modulação das respostas inflamatórias. É possível inferir que os efeitos analgésicos observados com o uso do antagonista A438079 são mediados, ao menos parcialmente, pela modulação das respostas inflamatórias. De forma relevante, o antagonista seletivo dos receptores P2X7, A438079, reduziu a produção de IL-1β e a migração de macrófagos a níveis basais, permitindo sugerir que estes são processos ligados e dependentes da ativação do receptor P2X7 durante o estabelecimento da CH.

Interessantemente, o presente trabalho mostrou, pela primeira vez, através de análise imunoistoquímica do tecido vesical, que há um aumento da expressão dos receptores P2X7 em camundongos com CH induzida por CYP. Este aumento poderia estar relacionado com a migração de células inflamatórias para a bexiga, tais como macrófagos e neutrófilos.

O conjunto de resultados obtidos no presente estudo indica que o receptor purinérgico P2X7 participa do processo inflamatório associado à CH induzida pela CYP em camundongos,

provavelmente pelo aumento da migração de neutrófilos e macrófagos e produção de citocinas pró-inflamatórias. Por sua vez, estes componentes inflamatórios podem ativar as vias nociceptivas periféricas e centrais diretamente ou, apenas facilitar o processo de nocicepção através da sensibilização dos terminais nociceptivos. É cabível propor que os receptores P2X7 seriam um alvo atrativo para o tratamento dos sintomas relacionados à CH severa, em pacientes sob tratamento com CYP. Ademais, o uso de antagonistas deste receptor poderia ser interessante, também, no tratamento dos sintomas da CH de etiologias diferentes. Por exemplo, na cistite intersticial, uma doença similar a CH, porém sem causa determinada, os receptores P2X7 poderiam representar um alvo terapêutico interessante.

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