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PAULA JULIANA BRIZUELA DE SEADI PEREIRA

CARACTERIZAÇÃO DO PAPEL DA FOSFATIDILINOSITOL-3 QUINASE γ NAS RESPOSTAS INFLAMATÓRIAS E NOCICEPTIVAS INDUZIDAS PELA TRIPSINA EM CAMUNDONGOS

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Dissertação apresentada como requisito para obtenção do grau 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.

Orientadora: Profª. Dra. Maria Martha Campos

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Aprovada em _____de_____de_____

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“O homem prudente deverá constantemente seguir o itinerário percorrido pelos grandes e imitar aqueles que mostraram-se excepcionais, a fim de que, caso o seu mérito ao deles não se iguale, possa ele ao menos recolher deste uma leve fragrância: procederá, assim agindo, como um prudente arqueiro, que, sabedor da distância que a qualidade do seu arco permite-lhe atingir, e , reconhecendo como demasiado longínquo o alvo escolhido, fixa a pontaria num ponto muito mais alto que o estipulado, esperando, não que a sua flecha alcance uma tamanha altura, mas poder, ajudado pela mira mais alta, atingir o ponto visado.”

Nicolau Maquiavel

RESUMO

Este estudo investigou os efeitos de inibidores seletivos para PI3Kγ nas respostas pruriceptivas, inflamatórias e nociceptivas induzidas por tripsina em camundongos. Os animais foram tratados por via oral com os inibidores seletivos de PI3Kγ AS605240 (1 a 30 mg/kg), AS041164 e AS252424 (ambos 1 mg/kg), 30 min antes dos experimentos. Em grupos separados, o AS605240 foi administrado por via intratecal (i.t.) ou intracerebroventricular (i.c.v.). Os grupos controles receberam solução salina nos mesmo esquemas de administração. Os efeitos da inibição da PI3K foram avaliados em diferentes modelos experimentais. O tratamento oral com AS605240 reduziu marcantemente o comportamento de coçar causado pela tripsina, enquanto o AS041164 e AS252424 não afetaram de forma significativa esse parâmetro. Além disso, o AS605240 (1 mg/kg) produziu uma inibição parcial, mas significativa do comportamento de coçar evocado pelo CP 48/80. De maneira interessante, a injeção i.t. e i.c.v. de AS605240 também reduziu o prurido causado pela tripsina. A administração oral de AS605240 foi efetiva em promover uma redução significativa e dose-dependente do edema de pata, produção de TNFα, bem como o recrutamento de neutrófilos induzido por tripsina. Do mesmo modo, o AS605240 (1 mg/kg) reduziu significativamente a nocicepção espontânea causada pela injeção de tripsina na pata dos animais. Por outro lado, a mesma dose de AS605240 não modificou a nocicepção induzida por capsaicina. Notavelmente, o AS605240 (1 mg/kg) previniu a imunopositividade para c-Fos e fosfo-Akt na medula espinhal dos camundongos injetados com tripsina tanto no dorso como na pata. Nossos dados sugerem que a inibição de PI3Kγ pode representar uma valiosa alternativa para o tratamento de condições inflamatórias e dolorosas, bem como o prurido.

Palavras-chave: Coceira; AS605240; PI3Kγ; tripsina; PAR-2; camundongo

ABSTRACT

This study investigated the effects of selective PI3Kγ inhibitors in the pruriceptive, inflammatory and nociceptive responses induced by trypsin in mice. The animals were orally treated with the selective PI3Kγ inhibitors AS605240 (1 to 30 mg/kg), AS041164 and AS252424 (both 1 mg/kg), 30 min beforehand. In separate groups, AS605240 was given by intrathecal (i.t.) or intracerebroventricular (i.c.v.) routes. The control groups received saline at the same schedules of administration. The effects of PI3K blocking were assessed in different experimental assays. The oral treatment with AS605240 produced a marked reduction of scratching behavior elicited by trypsin, whereas AS041164 and AS252424 administration failed to significantly affect this parameter. Moreover, AS605240 (1 mg/kg) was able to produce a partial, but significant inhibition of the scratching bouts elicited by CP 48/80. Interestingly, the i.c.v. and i.t. injection of AS605240 also reduced trypsin-induced itching.

The oral administration of AS605240 was found effective in producing a significant and dose-dependent reduction of trypsin-induced paw edema, TNFα production, as well as the neutrophil recruitment, according to MPO activity assessment. Likewise, oral AS605240 (1 mg/kg) promoted a significant reduction of spontaneous nociception induced by trypsin in the mouse paw. In contrast, the same dose of AS605240 did not significantly modify capsaicin-evoked nociception. Noteworthy, AS605240 (1 mg/kg) was effective in preventing c-Fos and phospho-Akt immunopositivity at the spinal cord of trypsin-injected mice, either into the dorsum or the paws. Our data suggests that PI3Kγ inhibitors might represent a valuable alternative for treating inflammatory and painful conditions, as well as pruritus.

Keywords: Itch; AS605240; PI3Kγ; trypsin; PAR-2; mice

ABREVIAÇÕES

5-HT – Serotonina

Akt/PKB - Proteína quinase B

AR - Artrite reumatoide

AS605240 - 5-quinoxalin-6-ilmetileno-tiazolidina-2,4-diona

ATP – Adenosina trifosfato

CGRP - Peptídeo relacionado com o gene da calcitonina

COX-2 – Ciclo-oxigenase 2

DPOC - Doença pulmonar obstrutiva crônica

GRKs - Quinases ligadas aos receptores acoplados à proteína G

GPCR - Receptores acoplados à proteína G

LES - Lupus eritematoso sistêmico

LY294002 - 2-(4-morfolinil)-8-fenil-4*H*-1-benzopiran-4-ona hidrocloreto

MAPK - Proteínas quinases ativadas por mitógenos

PARs - Receptores ativados por proteases

PI3K- Fosfatidilinositol-3-quinase

PIP_2 - Fosfatidilinositol-4,5-bifosfato

PIP_3 - Fosfatidilinositol-3,4,5- trifosfato

PKC - Proteína quinase C

PLC - Fofolipase C

SP - Substância P

SUMÁRIO

1. INTRODUÇÃO

As proteases são enzimas responsáveis pela quebra das ligações peptídicas e possuem diversas funções biológicas (Cottrell et al., 2003; Paszcuk et al., 2008). Por muito tempo, acreditou-se que eram apenas enzimas de degradação, mas, recentemente, tem sido demonstrado que as proteases podem atuar como moléculas de sinalização e desempenham importantes papéis na cascata de coagulação e no controle do ciclo celular (Cirino & Vergnolle, 2006; Cottrell et al., 2003; Paszcuk et al., 2008). Algumas proteases são geradas ou liberadas durante o trauma e estão envolvidas em processos patológicos como inflamação, dor, câncer e aterosclerose (Koshikawa et al., 1998; Vergnolle et al., 2001a). Grande parte das ações das proteases é mediada pelos receptores ativados por proteases (PARs – *proteinase-activated receptors*), que são receptores acoplados à proteína G (GPCR), com sete domínios transmembrana (Hollenberg, 2005; Sánchez-Hernández et al., 2008).

Quatro membros desta família, PAR-1, PAR-2, PAR-3 e PAR- 4, foram clonados até o momento (Hirano et al., 2005). De forma interessante, estes receptores são ativados de maneira irreversível por um único processo, que envolve o reconhecimento do receptor pela protease, seguidos da clivagem hidrolítica de um sítio específico localizado na alça N-terminal do receptor. Finalmente, ocorre a exposição de um novo sítio N-terminal que atua como o ligante (geralmente composto por seis ou mais aminoácidos). Uma vez exposto, o ligante endógeno interage com a alça extracelular do segundo domínio transmembrana do receptor, iniciando a ativação de diversas cascatas de sinalização intracelular (Déry et al., 1998; Paszcuk et al., 2008; Schmidlin & Bunnett, 2001; Vergnolle, 2005). A ativação destes receptores resulta em ligação a diferentes proteínas G. Os receptores mais estudados, PAR-1 e PAR-2, acoplam-se preferencialmente às proteínas G_i, G_q e $G_{12/13}$, promovendo direta ou

indiretamente, a estimulação de enzimas de sinalização como fofolipase Cβ (PLCβ), PLCγ, proteína quinase C (PKC), fosfatidilinositol-3-quinase (PI3K) e proteínas quinases ativadas por mitógenos (MAPK) (Costa et al., 2008; Kanke et al., 2005; Macfarlane et al., 2001; Paszcuk et al., 2008; Sabri et al., 2000; Schmidlin & Bunnett, 2001). Essa sinalização é altamente regulada e rapidamente finalizada por um mecanismo de dessensibilização e internalização do receptor. Depois de ativados, os receptores PARs sofrem fosforilação por quinases ligadas aos GPCRs (GRKs) e proteína quinase C. Minutos depois, as β-arrestinas são deslocadas do citosol até a membrana plasmática para interagirem com os receptores fosforilados, o que medeia o desacoplamento e a dessensibilização, pelo bloqueio da associação dos receptores com as proteínas G. As β-arrestinas acoplam os receptores PARs às clatrinas, que são necessárias para a endocitose destes. Depois de internalizados, os PARs são degradados pelos lisossomos (Paszcuk et al., 2008; Schmidlin & Bunnett, 2001). O segundo membro dessa família de receptores, PAR-2, pode ser ativado pela tripsina de fonte pancreática ou extra-pancreática, triptase e alguns fatores de coagulação como VIIa e Xa (Costa et al., 2008; Hollenberg, 2005; Kanke et al., 2005; Sánchez-Hernández et al., 2008).

Uma das serino-proteases mais bem caracterizadas é a tripsina. Ela é produzida em forma de zimogênio (tripsinogênio) nas células acinares do pâncreas, é secretada no duodeno e torna-se ativa devido à ação de enteroquinases, funcionando como uma enzima essencial para a digestão (Koshikawa et al., 1998; Paszcuk et al., 2008). Foram caracterizados quatro genes que codificam a tripsina em humanos: tripsinogênio I, II, III e IV. Tripsinogênios I, II e III são as principais formas presentes no suco pancreático, enquanto o IV é expresso no cérebro humano (Cottrell et al., 2003; Koshikawa et al., 1998). A tripsina é expressa em diversos tecidos e células, como por exemplo, células

endoteliais humanas ou células epiteliais da pele, esôfago, estômago, intestino, pulmão, rim, fígado e ducto biliar, bem como em leucócitos do baço e em células nervosas no cérebro, de humanos e camundongos (Koshikawa et al., 1998). Estudos anteriores mostraram que as proteases, entre elas a tripsina, são produzidas por células cancerígenas e regulam a proliferação e angiogênese. Ademais, acredita-se que sejam essenciais para a capacidade do tumor em invadir e degradar a matriz extracelular (Koivunen et al., 1991; Sánchez-Hernández et al., 2008). Entretanto, ainda se sabe pouco sobre as funções da tripsina em cada tipo de célula e em tecidos normais (Knecht et al., 2007; Koshikawa et al., 1998). Recentemente, tem sido demonstrado que a tripsina é capaz de evocar os sinais clássicos da resposta inflamatória, além de respostas nociceptivas e pruriginosas, especialmente através da ativação de receptores PAR-2 e da produção secundária de diversos mediadores da inflamação (Costa et al., 2008; Paszcuk et al., 2008).

Os receptores PAR-2 são amplamente expressos em diferentes tecidos e células, incluindo a pele, o trato gastrintestinal, pâncreas, pulmões, rins, bexiga, fígado, vias aéreas, próstata, ovários e olhos, e são encontrados em células epiteliais, células musculares lisas, fibroblastos, células endoteliais, células T, neutrófilos, mastócitos, eosinófilos, células tumorais e neurônios (Cottrell et al., 2003; Sánchez-Hernández et al., 2008; Schmidlin & Bunnett, 2001; Vergnolle, 2005; Vergnolle et al., 2001a). Embora a fisiologia e patofisiologia deste receptor ainda não estejam bem esclarecidas, vários autores têm sugerido seu potencial envolvimento no controle do tônus vascular (Déry et al., 1998; Macfarlane et al., 2001; Vergnolle et al., 2001b).

Diversos estudos apontam um papel pró-inflamatório para a ativação do PAR-2. A estimulação desta proteína leva ao aumento da permeabilidade vascular, vasodilatação dependente de óxido nítrico, indução de extravasamento de proteínas

plasmáticas, infiltração de leucócitos e secreção de citocinas pró-inflamatórias (Schmidlin & Bunnett, 2001; Vergnolle et al., 2001b). A ativação dos receptores PAR-2 nos principais neurônios espinhais aferentes estimula a secreção de substância P (SP) e do peptídeo relacionado com o gene da calcitonina (CGRP), a partir de suas projeções em tecidos periféricos e medula espinhal, causando inflamação neurogênica (Cottrell et al., 2003). Os receptores PAR-2 parecem desempenhar um papel mais importante em situações patológicas do que em condições fisiológicas, estando envolvidos tanto no início, quanto na manutenção de processos inflamatórios e dolorosos (Costa et al., 2008; Hirano et al., 2005; Hollenberg, 2005; Kanke et al., 2005). Ademais, este tipo de receptor tem sido apontado como um efetor importante de processos pruriginosos (Costa et al., 2008).

As respostas nociceptivas e inflamatórias evocadas pela tripsina *in vivo* parecem depender da ativação direta de nociceptores, da intensa infiltração de neutrófilos e da liberação de diferentes mediadores da inflamação, incluindo bradicinina, prostanoides, neuropeptídeos, serotonina (5-HT) e histamina (Vergnolle et al., 2001a; Vergnolle et al., 1999; Vergnolle et al., 2001b). Paszcuk e colaboradores (2008) demonstraram que as respostas edematogênica e nociceptiva induzidas pela tripsina na pata de camundongos parecem constituir eventos multimediados, envolvendo a ação de diversos mediadores e a ativação de diferentes receptores, entre eles o PAR-2. A tripsina também é capaz de promover resposta pruriginosa através da ativação dos receptores PAR-2 localizados nas fibras C, fibroblastos, queratinócitos ou mastócitos, que leva à liberação de diferentes moléculas, tais como proteases, 5-HT, histamina, SP, CGRP, prostaglandinas (via ativação de COX-2) e cininas. Estes mediadores atuam em conjunto para ativar e/ou sensibilizar nervos sensoriais, transmitindo a sensação de prurido para estruturas centrais (Costa et al., 2008). Entretanto, pouco se sabe acerca das vias de sinalização

intracelulares ativadas pela estimulação do PAR-2, durante alterações inflamatórias, nociceptivas e pruriginosas.

As fosfatidilinositol-3-quinases (PI3Ks) são um grupo de enzimas que fosforilam o fosfatidilinositol-4,5-bifosfato (PIP_2) na porção 3´-OH do anel inositol, dando origem ao segundo mensageiro fosfatidilinositol-3,4,5- trifosfato (PIP_3), que é essencial para a translocação de Akt (também conhecida como PKB) para a membrana plasmática, onde a mesma também é fosforilada. A ativação de Akt medeia respostas celulares como proliferação, sobrevivência, motilidade e metabolismo (Kong & Yamori, 2007; Marone et al., 2008; Ohashi & Woodgett, 2005; Rommel et al., 2007; Roy et al., 2009; Zhuang et al., 2004). A família das PI3Ks é dividida em três classes (I,II e III). A classe I, por sua vez, é dividida em duas subclasses: IA e IB. A subclasse IA que compreende as isoformas PI3Kα, PI3Kβ e PI3Kδ, é ativada através de receptores tirosina-quinase pela ligação de hormônios, fatores de crescimento, citocinas, integrinas e outros estímulos extracelulares. A subclasse IB possui apenas um membro, a isoforma PI3Kγ, que é ativada por receptores acoplados à proteína G, através da interação das subunidades regulatórias com as subunidades βγ das proteínas G. A PI3Kα e PI3Kβ são expressas em todas as células e regulam uma variedade de funções, incluindo sobrevivência e proliferação celular. Assim como a PI3Kδ, a PI3Kγ é expressa em leucócitos e regula respostas imunes (quimiotaxia de leucócitos e degranulação de mastócitos), embora esta última também possa ser encontrada no endotélio, coração e cérebro (Barber et al., 2005; Camps et al., 2005; Ferrandi et al., 2007; Fröjdö et al., 2007; Ito et al., 2007 ; Ohashi & Woodgett, 2005; Pomel et al., 2006; Rommel et al., 2007; Rückle et al., 2006). O aumento constitutivo da sinalização da PI3K pode ter efeito deletério nas células, levando à proliferação descontrolada, migração facilitada e adesão independente de crescimento. Estes eventos favorecem não somente a formação

de tumores malignos, mas também o desenvolvimento de inflamação crônica, alergia, diabetes, problemas cardiovasculares e doenças autoimunes (Gharbi et al., 2007; Ito et al., 2007 ; Marone et al., 2008).

Diversos estudos têm sugerido que a inibição da PI3K pode ter um potencial terapêutico para doenças como artrite reumatoide (AR), lupus eritematoso sistêmico (LES), psoríase, doença pulmonar obstrutiva crônica (DPOC), asma e esclerose múltipla (Barber et al., 2005; Camps et al., 2005; Marone et al., 2008; Medina-Tato et al., 2007). A inibição da isoforma PI3Kγ constitui um novo conceito para o controle de alterações presentes em doenças inflamatórias e autoimunes (Ferrandi et al., 2007; Rückle et al., 2006). Existem produtos naturais que são capazes de inibir de maneira não seletiva a PI3K, entre eles está o wortmannin que é um produto isolado do fungo *Penicillium funiculosum,* a demetoxiviridina que também é um produto isolado de fungo (*Nodulisporium hinnuleum*) e o resveratrol, que é um polifenol presente em uvas, vinho tinto e amendoins (Fröjdö et al., 2007; Marone et al., 2008; Roy et al., 2009). No início dos anos 90, começaram a ser desenvolvidos os inibidores sintéticos da PI3K de primeira geração. O primeiro a ser sintetizado foi o LY294002 (2-(4-morfolinil)-8-fenil-4*H*-1-benzopiran-4-ona hidrocloreto), um inibidor reversível derivado da quercitina. As fracas propriedades biofarmacêuticas e a falta de seletividade para isoformas destes inibidores de primeira geração levaram à procura de moléculas mais seletivas, principalmente para a isoforma PI3Kγ, que representa um alvo farmacológico importante e promissor para o tratamento de doenças inflamatórias (Ferrandi et al., 2007; Gharbi et al., 2007; Ito et al., 2007 ; Marone et al., 2008; Pomel et al., 2006). No processo de desenvolvimento dos inibidores da PI3K de segunda geração, diversos tipos químicos têm sido identificados. Estes inibidores são ativos contra várias PI3Ks e são úteis para explorar a importância destas enzimas (Marone et al., 2008).

O AS605240 (5-quinoxalin-6-ilmetileno-tiazolidina-2,4-diona) é um inibidor competitivo de ATP, com baixo peso molecular, específico para PI3Kγ, ativo por via oral, que apresenta alta potência e tem ótimas propriedades de permeabilidade celular (Camps et al., 2005). Estudos recentes têm demonstrado que a inibição da PI3Kγ, através da utilização do AS605240, bloqueia a progressão de dano e inflamação nas articulações, em modelos de artrite reumatoide em camundongos e, aumenta a sobrevivência de camundongos num modelo de lupus eritematoso sistêmico, diminuindo a deposição de imunocomplexos, proteinúria e níveis de auto-anticorpos (Barber et al., 2005; Camps et al., 2005). Em ambos os estudos não foram observados efeitos adversos ao tratamento. De maneira interessante, Fougerat e colaboradores (2008) demonstraram que a administração de AS605240 foi capaz de produzir uma redução importante das lesões ateroscleróticas em modelos de aterosclerose *in vivo*.

A busca por novas terapias anti-inflamatórias tem-se tornado imprescindível, principalmente após os episódios de toxicidade com os inibidores de COX-2. Por isso, a identificação e o desenvolvimento de novos agentes para tratar processos inflamatórios possuem enorme interesse para a medicina (Fougerat et al., 2008; Ohashi & Woodgett, 2005).

2. OBJETIVOS

2.1. Objetivo Geral

O presente trabalho teve como objetivo investigar o envolvimento da PI3Kγ em diferentes efeitos mediados pela tripsina *in vivo*, através do emprego de técnicas farmacológicas, bioquímicas e de biologia molecular.

2.2. Objetivos Específicos

- Investigar o efeito do bloqueio farmacológico da PI3Kγ, sobre a resposta edematogênica causada pela tripsina em camundongos.
- Avaliar o efeito do bloqueio seletivo da PI3Kγ sobre as respostas nociceptivas e pruriceptivas induzidas pela tripsina em camundongos.
- Analisar o efeito do tratamento com inibidor seletivo da PI3Kγ sobre a migração de células inflamatórias após a injeção de tripsina.
- Determinar os efeitos do bloqueio farmacológico da PI3Kγ sobre a produção de TNFα induzida pela tripsina, através de ensaios de ELISA.
- Avaliar a fosforilação da AKT e a expressão de c-Fos em estruturas centrais, após a aplicação de tripsina em camundongos, através de imunohistoquímica.

3. MANUSCRITO DO TRABALHO EXPERIMENTAL

Os resultados do presente trabalho foram submetidos à revista PAIN®, fator de impacto 5.371 (2009).

Inhibition of phosphatidylinositol-3 kinase γ reduces pruriceptive, inflammatory and nociceptive responses induced by trypsin in mice

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Abstract

This study investigated the effects of selective PI3Kγ inhibitors in the pruriceptive, inflammatory and nociceptive responses induced by trypsin in mice. The animals were orally treated with the selective PI3Kγ inhibitors AS605240 (1 to 30 mg/kg), AS041164 and AS252424 (both 1 mg/kg), 30 min beforehand. In separate groups, AS605240 was given by intrathecal (i.t.) or intracerebroventricular (i.c.v.) routes. The control groups received saline at the same schedules. The effects of PI3K blocking were assessed in different experimental assays. The oral treatment with AS605240 produced a marked reduction of scratching behavior elicited by trypsin, whereas AS041164 and AS252424 administration failed to significantly affect this parameter. Moreover, AS605240 (1 mg/kg) was able to produce a partial, but significant inhibition of the scratching bouts elicited by CP 48/80. Interestingly, the i.c.v. and i.t. injection of AS605240 also reduced trypsin-induced itching.

The oral administration of AS605240 was found effective in producing a significant and dose-dependent reduction of trypsin-induced paw edema, TNFα production, as well as the neutrophil recruitment, according to MPO activity assessment. Likewise, oral AS605240 (1 mg/kg) promoted a significant reduction of spontaneous nociception induced by trypsin in the mouse paw. In contrast, the same dose of AS605240 did not significantly modify capsaicin-evoked nociception. Noteworthy, AS605240 (1 mg/kg) was effective in preventing c-Fos and phospho-Akt immunopositivity at the spinal cord of trypsin-injected mice, either into the dorsum or the paws. To conclude, PI3Kγ inhibitors might well represent a valuable alternative for treating inflammatory and painful conditions, as well as pruritus.

Keywords: Itch, AS605240, PI3Kγ, trypsin, PAR-2, mice

Introduction

The phosphatidylinositol 3-kinases (PI3Ks) belong to a family of enzymes that phosphorylate the inositol ring at the 3´- hydroxyl position of phosphatidylinositol and phosphoinositides. PI3Ks are divided into three classes (I, II and III) according to their structure and substrate specificity [7,31]. Class I PI3Ks are separated into two subclasses: IA and IB. Both catalyze the phospholylation of phosphatidylinositol-4,5-bisphosphate (PIP2) generating phosphatidylinositol-3,4,5-trisphosphate (PIP3). This molecule is a lipid second messenger and is essential for the recruitment of protein serine/threonine kinase AKT (also known as PKB) to the plasma membrane, where it is phospholylated and activated by phosphoinositide-dependent kinase 1 (PDK1) and mammalian target of rapamycin complex 2 (mTORC2). Once activated, AKT mediates several functions such as cell proliferation, survival, motility and metabolism [22,32]. Class IA (consisting of PI3Kα, PI3Kβ and PI3Kδ isoforms) are often activated by hormones, cytokines, integrins, growth factors and other extracellular stimuli via the tyrosine kinase receptors; differently, the only member of class IB - PI3Kγ - is activated by G-protein-coupled receptors (GPCRs) [9,33]. Whereas PI3Kα and PI3Kβ are ubiquitously expressed and regulate functions such as cell proliferation and survival, PI3Kδ and PI3Kγ are mainly expressed in the hematopoietic system and mediate immune responses. PI3Kγ can also be found in endothelium, heart and brain [8,33]. In addition, it has been described that the PI3Kγ isoform plays a pivotal role in inflammation, and it is involved in allergy, cardiovascular disorders, development of chronic inflammation and autoimmune diseases [3,9,22]. Therefore, PI3Kγ inhibition might represent an interesting alternative to control autoimmune and inflammatory diseases [8,33].

Recently, proteinases have been described as signaling molecules that play important roles in functions such as the coagulation cascade and control of the cell cycle [4,6]. Certain proteinases are released or generated during trauma and are involved in pathological processes including inflammation, pain, cancer and atherosclerosis [19,37]. Proteinases exert most part of their effects by activating a family of specific GPCRs, named proteinase-activated receptors (PARs) [6,37]. Trypsin is one of the best characterized serine proteinases. It is widely expressed in vascular endothelial cells of humans or epithelial cells of the skin, esophagus, stomach, intestines, lungs, kidneys, liver and extrahepatic bile duct, as well as in spleen and neurons, from mammalians [19,28]. There is evidence that proteinases, including trypsin, are produced by malignant tumor cells and they regulate proliferation, angiogenesis, invasion and metastasis [34]. In addition, it has been demonstrated that trypsin is able to evoke the classic signals of inflammation, including nociceptive and pruriceptive responses, mainly by the activation of proteinase-activated receptor 2 (PAR-2), and the secondary production of several inflammatory mediators [5,28]

In this study, we sought to investigate the role of PI3Kγ in the pruriceptive, inflammatory and nociceptive responses induced by trypsin in mice, with efforts to compare the effects of different selective inhibitors of this isoform. We have also aimed to determine the relevance of PI3Kγ for the activation of spinal nociceptive and pruriceptive pathways, following the peripheral injection of trypsin.

Methods

Animals

Male *Swiss* mice (6-8 per group, 25-30 g) were used in this study. The animals were housed in groups of eight and maintained in controlled temperature (22 ± 1 ºC) and humidity (60 - 70 %), under a 12 h light-dark cycle (lights on 07:00 AM). Food and water were available *ad libitum*. Animals were acclimatized to the laboratory for at least 1 h before testing and were used only once throughout the experiments. All the tests were performed between 7:00 AM and 7:00 PM. The experimental procedures reported in this manuscript followed the "Principles of Laboratory Animal Care" from NIH publication No. 85-23, and were approved by the Institutional Animal Ethics Committee (protocol number: 09/00101). The number of animals and intensity of noxious stimuli used were the minimum necessary to demonstrate the consistent effects of the drug treatments.

Induction of scratching behavior

The experimental protocols were carried out according to the method described by Costa et al. [5]. Two days before the experiments, mouse dorsum was shaved. On the day of experiments, the animals were individually placed into glass cylinders for at least 30 min to acclimatize to the new environment. After this period, each mouse was briefly removed and received an intradermal (i.d.) injection of saline (50 µl) containing trypsin (200 µg/site) or the mast cell depletor compound 48/80 (10 µg/site). Immediately after injection, the animals were returned to the cylinders. The animals were observed for 40 min, and the scratching behavior was measured as the number of scratches with forepaws and hindpaws close to the injected site. Scratching behind the ears, but not on the face, was also counted. The results were expressed as the total number of scratches

in 40 min. At least two mice (control and treated) were observed simultaneously in each experimental session.

Paw edema evoked by trypsin

The procedure used was similar to the method described by Paszcuk et al. [28]. Animals were lightly anesthetized with isoflurane and edema was induced in the right hindpaw by a 50 µl intraplantar (i.pl.) injection of saline containing trypsin (30 µg/paw). The left paw received the same volume of saline and it was used as the control. Edema was determined by a plethysmometer (Ugo Basile) at different periods of time (0.5 to 6 h) after injection of trypsin. Edema was expressed in µl, as the difference between the volume of the right and the left paws.

Spontaneous nociception

After an adaptation period of 30 min, each animal received a 20 µl i.pl. injection of trypsin (300 µg/paw) or capsaicin (1.6 µg/paw) into the right hindpaw. The mice were observed for 10 and 5 min following trypsin or capsaicin injection, respectively [18,28]. The amount of time (in seconds) spent licking and/or biting the injected paw was recorded, and it was considered as indicative of spontaneous nociception. At least two mice (control and treated) were observed simultaneously in each experimental session.

Neutrophil migration induced by trypsin: MPO assay

Neutrophil recruitment to the mouse paw was quantified indirectly by tissue myeloperoxidase (MPO) activity, according to the method described by Paszcuk et al. [28], with minor modifications. Mice received a 50 µl i.pl. injection of trypsin (30 µg/paw) into the right hindpaw. Three hours after trypsin injection the animals were euthanized, the subcutaneous tissue of the paws was immediately removed and frozen. Saline-treated paws were used as negative controls. The tissues were homogenized at 5% (w/v) in EDTA/NaCl buffer (pH 4.7) and centrifuged at 4400 ×g for 15 min at 4 °C. The pellet was re-suspended in 0.5% hexadecyltrimethyl ammonium bromide buffer (pH 5.4), and the samples were frozen and thawed three times in liquid nitrogen. Upon thawing, the samples were re-centrifuged (4400 ×g, 15 min, 4 °C), and 25 µl of the supernatant was used for the MPO assay. The enzymatic reaction was assessed with 1.6 mM tetramethylbenzidine, 80 mM Na_3PO_4, and 0.3 mM hydrogen peroxide. The absorbance was measured at 650 nm, and the results are expressed as Optical Density (OD) per milligram of tissue.

Determination of TNFα levels in mouse paw

The tissue levels of tumor necrosis factor α (TNFα) were evaluated by ELISA. For this purpose, animals received a 50 µl i.pl. injection of trypsin (30 µg/paw) into the right hindpaw. After 6 h, the animals were euthanized; the subcutaneous tissue of the paws was immediately removed and frozen at -20 ºC. Saline-treated paws were used as negative controls. The tissues were homogenized in phosphate buffered saline containing: NaCl 0.4 M, PMSF 0.1 M, EDTA 10 mM, 0.05% Tween 20, 0.5% BSA and 2 µg/ml of aprotinin A. The samples were centrifuged at 5000 rpm for 10 min at 4 ºC, and the supernatant were used for the assay. The TNFα levels were measured using an

ELISA kit, according to the recommendations of the supplier (DuoSet Kit; R&D Systems, Minneapolis, MN). The results are expressed in pg/mg of tissue.

Immunohistochemistry for phospho-Akt and c-Fos

The method described by Medeiros et al.[23] was used for imunohistochemistry analysis. Trypsin-injected animals (control group and treated with AS605240) were euthanized and lumbar spinal cords (L3-L6) were collected, fixed and embedded in paraffin. Saline-treated mice were used as negative controls. Two different experimental protocols were used: (i) saline or trypsin were injected in the dorsum and the samples were collected 10 min later; (ii) saline or trypsin were injected into the right hindpaw and the spinal cords or brains were collected at 5 min.

Detection of phospho-Akt and c-Fos was assessed on paraffin tissue sections, using the polyclonal rabbit anti-phospho-Akt (1: 500 R&D Systems, Minneapolis, MN) and polyclonal rabbit anti-c-Fos (1:700, Abcam), respectively. High-temperature antigen retrieval was performed by immersion of the slides in a water bath at 95–98°C in 10 mM trisodium citrate buffer, pH 6.0, for 45 min. The nonspecific binding was blocked by incubating sections for 1 h with goat normal serum diluted in PBS. After overnight incubation at 4°C with primary antibodies, the slides were washed with PBS and incubated with the secondary antibody Envision plus (Dako Cytomation), ready to use, for 1 h at room temperature. The sections were washed in PBS, and the visualization was completed by using 3,3'-diaminobenzidine (Dako Cytomation) in chromogen solution and counterstained lightly with Harris's hematoxylin solution. Immunoreactivity was visualized as a brown staining in the cell nuclei. Images were taken with a microscope (Eclipse 50i; Nikon) and digital sight camera (DS-5M-L1;

Nikon). Control and experimental tissues were placed on the same slide and processed under the same conditions. Settings for image acquisition were identical for control and experimental samples. For each mouse, we have obtained at least three images (one per section). Digitized, 8-bit images were transferred to a computer, and the average pixel intensity was calculated for each image using NIH ImageJ 1.36b imaging software (National Institutes of Health, Maryland, EUA).

Pharmacological treatments

In order to characterize the role of PI3Kγ in the inflammatory, nociceptive and pruritogenic responses induced by trypsin**,** the animals were treated orally with the selective PI3Kγ inhibitors AS605240 (0.3 to 30 mg/kg., 30 min), AS041164 and AS252424 (both 1 mg/kg., 30 min). The effects of AS605240 were also evaluated on the scratching behavior caused by compound 48/80, and spontaneous nociception elicited by capsaicin.

To further assess the sites of action of AS605240, in separate sets of experiments, the compound was administered by intrathecal (i.t.) or intracerebroventricular (i.c.v.) routes. The i.t. injections were performed in conscious animals to avoid possible anesthetic interference, according to the method described by [14], with some modifications. The needle connected to a microsyringe by a polyethylene tubing was introduced through the skin, and a volume of 5 µl of vehicle (control) or AS605240 (5 µg/site) was injected between the L5 and L6 vertebral spaces. For i.c.v. injections, the animals were slightly anesthetized by inhalation with isoflurane, and a free-hand injection of a 5-µl volume of AS605240 (3 and 5 µg/site) or vehicle was injected directly into left ventricle of the brain, as described previously by

[21]. The doses of inhibitors were selected on the basis of literature data and pilot experiments [2,3].

Statistical analysis

The results are presented as the mean ± standard error mean of 4 to 8 animals per group. The percentages of inhibition were calculated as the mean of inhibitions obtained for each individual experiment or, on the basis of the area under the curve (paw edema model). Statistical comparison of the data was performed by one-way analysis of variance (ANOVA) followed by Dunnet test or Tukey test. *P*-values less than 0.05 ($P < 0.05$) were considered significant.

Drugs and reagents

The following drugs and reagents were used: 5-quinoxalin-6-ylmethylene-thiazolidine-2,4-dione (AS605240) and 5-[5-(4-fluoro-2-hydroxy-phenyl)-furan-2-ylmethylene]-thiazolidine-2,4-dione (AS252424) were purchased from Cayman Chemical Company (Ann Harbor, MI, USA); 5-(Benzo[1]dioxol-5-ylmethylene)-thiazolidine-2,4-dione (AS041164) was supplied by Calbiochem (San Diego, CA, USA); trypsin (from bovine pancreas), compound 48/80, capsaicin, carboxymethylcellulose, ethylene diamine tetracetic acid (EDTA), hexadecyltrimethyl ammonium bromide (HTAB), tetramethylbenzidine, phenylmethylsulfonyl fluoride, benzamethonium chloride, and aprotinin A all came from Sigma-Aldrich (St. Louis, MO, USA); Na_3PO_4, NaCl, glucose and $NaPO_4$ (all from Merk, Haar, Germany); hydrogen peroxide and Tween 20 (Vetec, RJ, Brazil); bovine serum albumin (BSA)

from Promega (Madison,WI, USA); anti-c-Fos (Abcam, Cambridge, MA, USA); anti-phospho-Akt and TNFα DuoSet kit (R&D Systems, Minneapolis, MN, USA). Trypsin, compound 48/80 and AS-252424 (provided in ethanol at the concentration of 1mg/ml) were diluted in saline solution (0.9% NaCl), capsaicin was initially diluted in 5% ethanol solution (in saline), AS605240 was dissolved in vehicle (0.5% carboxymethylcellulose/ 0.25% Tween 20), AS041164 was diluted in 2% DMSO (in saline). The control groups received the respective vehicle used to dissolve the drugs. The final concentrations of solvents never exceeded 2 % and did not display any significant effect *per se*.

Results

Effect of the treatment with selective PI3Kγ inhibitors on the scratching behavior evoked by trypsin or compound 48/80 in mice

In this set of experiments, we investigated whether the inhibition of PI3Kγ might interfere with the pruriceptive response evoked by trypsin, in comparison to the scratching caused by the compound 48/80. For this goal, three selective inhibitors of PI3Kγ were tested. As depicted in Figure 1A, the oral treatment with the selective PI3Kγ inhibitor AS605240 (1, 3 and 10 mg/kg, 30 min) promoted a significant and marked inhibition of trypsin-elicited scratching behavior, with reduction percentages of 63 ± 8%, 59 ± 11% and 55 ± 8%, respectively. On the other hand, the administration of AS605240 at the dose of 0.3 mg/kg did not display any significant reduction of the scratching behavior caused by trypsin. When the animals were orally treated with AS041164 and AS252424 (both 1 mg/kg, 30 min) the scratching response was partially diminished; however, this effect was not significant (Figure 1B). Moreover, the effect of AS605240 was evaluated in the scratching behavior induced by the mast cell depletor compound 48/80. Figure 1C shows that AS605240 (1 mg/kg, 30 min), given by oral route, produced a partial, but significant inhibition of the scratching bouts elicited by compound 48/80 (25 ± 6% of inhibition). The i.c.v. injection of AS605240 (3 μg/site) showed a slight, but not significant decrease of the scratching bouts. Interestingly, the administration of AS605240 by i.c.v. route at the dose of 5 μg/site was able to significantly reduce the scratching behavior induced by trypsin, with 56 ± 17% of inhibition (Figure 1D). In contrast, as seen in Figure 1E,the i.t. treatment with 5 μg/site of AS605240 leads to a partial, although not significant inhibition of trypsin-evoked scratches.

Effect of the treatment with the selective PI3Kγinhibitor AS605240 on the paw edema induced by trypsin in mice

To assess the role of PI3Kγ in the inflammatory response induced by trypsin, a selective inhibitor for this isoform was used. The intraplantar injection of trypsin (30 µg/paw) caused a marked- and time-dependent paw edema in mice (area under curve = 649 ± 26). The administration of AS605240 (1, 3, 10 and 30 mg/kg.), given 30 min before, produced a significant and dose-dependent reduction of trypsin-induced paw edema formation (Figure 2A to D). According to the calculation of the areas under the curve, as shown in Figure 2 (Inbox), the obtained percentages of reduction were 24 ± 7%, 46 ± 3%, 40 ± 7% and 42 ± 3%, for the doses of 1, 3, 10 and 30 mg/kg, respectively.

Effect of the treatment with the selective PI3Kγ inhibitor AS605240 on the spontaneous nociception caused by trypsin or capsaicin in mice

Next, we analyzed whether PI3Kγ inhibition is capable of reducing the spontaneous nociception induced by trypsin. The oral treatment with AS605240 (0.3 and 3 mg/kg, 30 min) was not able to affect the trypsin-elicited spontaneous nociception in a significant manner (Figure 3A). Interestingly, when mice were treated with AS605240 at the dose of 1 mg/kg, the spontaneous nociceptive response was strongly and significantly decreased, with 61 ± 7% of inhibition (Figure 3A). Furthermore, the i.c.v. injection of AS605240 (5 µg/site) practically abolished the spontaneous nociception induced by trypsin (Figure 3B). This response was statistically significant, with an inhibition percentage of 99 ± 0.8 %. Nevertheless, when 5 µg/site of AS605240 was administered by i.t. route, the nociceptive response was only slightly reduced, as shown in Figure C. The spontaneous nociception caused by capsaicin was also assessed

in order to verify the effect of AS605240 in this model. As shown in Figure 3D, the administration of AS605240 (1 mg/kg, 30 min) by oral route did not modify the capsaicin-evoked nociceptive response.

Effect of AS605240 on neutrophil migration induced by trypsin in mice

In order to confirm the role of PI3Kγ in the inflammatory response induced by trypsin, the MPO activity in the mouse paw was measured. As seen in Figure 4, the animals administered orally with the selective PI3Kγ inhibitor AS605240 (3 mg/kg, 30 min) showed a partial, but not significant reduction of MPO activity. On the other hand, the treatment with AS605240 at the dose of 10 mg/kg caused a marked and significant inhibition of MPO activity (55 ± 4%, Figure 4A).

Effect of AS605240 on TNFα levels increased by trypsin in mice

Another parameter analyzed was the production of tumor necrosis factor α (TNFα) in the mouse paw tissue. As shown in Figure 4B, trypsin injection into the mouse paw promoted a visble increase in TNFα levels, while the treatment with AS605240 (10 mg/kg, 30 min) reduced this parameter to the levels observed in the saline control group. However, this effect was not statistically significant.

Effects of the treatment with AS605240 on c-Fos expression and phospho-Akt activation in mice

To investigate whether PI3Kγ inhibition would be able to reduce the expression of the neuronal activation marker c-Fos, we have performed an immunohistochemistry analysis of lumbar spinal cord sections. For the dorsum injection, discrete c-Fos immunolabeling was detected in the spinal cord of saline-injected animals (Figure 5A). However, in the trypsin-injected group (200 μg/site), the pattern of expression was significantly increased when compared to the control saline group (Figure 5B). Of high interest, the pre-treatment with AS605240 (1 mg/kg, 30 min), by oral route, was able to strongly reduce the c-Fos immunolabeling (Figure 5C). This effect occurred in a significant manner, with an inhibition percentage of 93 ± 2 % (Figure 5D).

For the i.pl. administration, it was possible to observe a slight c-Fos immunostaining at the spinal cord of mice injected with saline (Figure 6A).In contrast, trypsin injection (300 μg/site) caused an increase of c-Fos immunoreactivity (Figure 6B). Indeed, the oral treatment with AS605240 (1 mg/kg, 30 min) was capable of reducing c-Fos expression (Figure 6C), although this effect was not statistically significant (Figure 6D).

In order to verify whether inhibition of PI3Kγ could prevent Akt phosphorylation at spinal levels, immunohistochemistry analysis to determine the phosphorylation of this protein was performed on spinal cord sections. As depicted in Figure 7A, slight phospho-Akt immunoreactivity was observed in the spinal cord of mice injected with saline into the dorsum. On the other hand, the injection of trypsin promoted a significant elevation of phospho-Akt immunolabeling when compared to the saline group (Figure7B). Notably, the immunostaining for phospho-Akt was partially

reduced when AS605240 (1 mg/kg, 30 min) was orally administered (Figure 7C), although this reduction was not significant, as seen in Figure 7D.

When mice were injected with saline into the right hindpaw, phospho-Akt immunostaining was detected (Figure 8A). As expected, trypsin injection increased the immunolabeling for phospho-Akt (Figure 8B). Interestingly, as shown in Figure 8C, the phospho-Akt immunoreactivity was significantly reduced by the oral treatment with AS605240 (1 mg/kg, 30 min). The inhibition percentage was 35 ± 4 % (Figure 8D).

Discussion

PI3Kγ belongs to class IB of PI3K family, which is activated via GPCRs, and is preferentially expressed in the hematopoietic system. It has been reported, especially in the past 10 years, that γ-isoform plays a pivotal role in inflammation [10,29]. This fact is mainly due to the ability of PI3Kγ to activate and recruit leukocytes during inflammatory responses [27].

The present study was designed to investigate the involvement of PI3Kγ in the pruriceptive, inflammatory and nociceptive responses induced by trypsin, when injected into the mouse paw or dorsum. Some efforts have also been made to determine the possible anatomical sites related to PI3Kγ activation following trypsin injection.

Trypsin is a serine proteinase and the preferential activator of G protein-coupled receptor PAR-2 [6]. Recently, it has been described that trypsin is capable of inducing paw inflammation and nociception, being also implicated in cancer pain in mice, mostly by activation of PAR-2 [20,28]. Furthermore, trypsin might cause pruritus, also by PAR-2 activation, and it can be used as a reproducible model to evaluate itching [5]. Our results show, for the first time, that oral treatment with the potent selective PI3Kγ inhibitor AS605240, strongly and significantly reduced trypsin-elicited scratching behavior. Conversely, two other selective inhibitors of PI3Kγ AS041164 and AS252424 displayed only a partial diminution of the pruriceptive response caused by trypsin. As the 1 mg/kg dose of AS605240 provided the best effects in the model of scratching behavior, we decided to evaluate the same dose for the other two selective inhibitors of PI3Kγ. However, our data suggest that AS605240 seems to be more effective than AS041164 and AS252424, at least in the scratching behavior paradigm. In fact, Ferrandi et al. [8], have demonstrated only a partial effect for AS041164 in the rat paw edema model induced by carrageenan application.

The compound 48/80 is a potent mast cell degranulator, which causes the release of several mediators from these cells, such as histamine, serotonin, proteinases and prostanoids. It is also well characterized that compound 48/80 induces pruritus in both, humans and mice [5,16]. In order to determine whether AS605240 might modulate the pruritus elicited by other agents, we assessed the effects of this inhibitor on compound 48/80-induced itching. Confirming literature data [35], mice exhibited an intense scratching behavior when injected with compound 48/80. However, this effect was only partially diminished by pre-treating animals with 1 mg/kg of AS605240, indicating a minor participation of PI3Kγ pathway activation in mast cell-mediated pruritus. This reduction might well be related to an interference with compound 48/80-induced protease release.

To further assess the mechanisms underlying the inhibitory effects of AS605240 in trypsin scratching model, we have used two different approaches. Firstly, we have tested this inhibitor by either i.c.v. or i.t. routes. The i.c.v. injection was capable of markedly reducing trypsin-induced scratching behavior. These results are supported by data demonstrating that PI3Kγ is present and can be activated in the mouse brain [25,27]. In contrast, for the i.t. treatment, the itching response was only partially attenuated. Thereby, our results might imply that brain, rather than spinal PI3Kγ activation likely displays an important role in the trypsin-evoked pruritus.

As another strategy, we have performed immunohistochemical analysis to determine the ability of the PI3Kγ inhibitor AS605240 in modulating central mechanisms involved in sensorial transmission of itching. Several studies have reported that pruritogenic agents augment the activation of c-Fos expression in the dorsal horn neurons of rats and mice [15,17,26]. In our study, trypsin injection into the mouse dorsum caused a striking elevation of c-Fos immunoreactivity in the spinal cord. In fact,

it has been recently demonstrated that intradermal injection of the selective PAR-2 agonist SLIGRL-NH_2 produced a significant increase of spinal c-Fos immunostaining [1]. Of interest, we demonstrate herein that oral administration of AS605240 promoted a strong and significant inhibition of c-Fos immunolabeling induced by trypsin. The spinal modulation of c-Fos might represent one of the mechanisms underlying the anti-pruritic effects of AS605240 in the trypsin model.

Xu et al. [39] have shown that activation of PI3K/Akt signaling pathway in the rat spinal cord contributes to the development of neuropathic pain. Thus, based on this finding, and in our own results of c-Fos expression, we decided to investigate whether spinal PI3Kγ activation participates in itching induced by trypsin. The injection of trypsin into the mouse dorsum resulted in increased phospho-Akt immunostaining in the spinal cord. Additionally, we show that oral treatment with AS605240 was able to decrease the phospho-Akt immunolabeling in the trypsin-induced pruritus model. Our data suggest that activation of PI3Kγ might be involved in transmission of pruriceptive processes.

Considering its pivotal role in mechanisms underlying inflammation, PI3Kγ has recently become a promising target for the treatment of autoimmune and inflammatory diseases [10,38]. A series of studies in mouse models have shown that PI3Kγ inhibition by AS605240 was effective on autoimmune and inflammatory conditions, including rheumatoid arthritis, systemic lupus, atherosclerosis, hepatic injury and intestinal bowel diseases [2,3,9,29,38].

In this study, we observed that AS605240, given orally, was able to modify inflammatory responses, producing a marked reduction of paw edema formation, neutrophil infiltration, and TNFα production caused by trypsin injection into the mouse paw. Our results are consistent with those of Peng et al.[29] reporting that AS605240

treatment decreased neutrophil migration and TNFα levels in a murine model of colitis. In addition, Camps et al.[3] showed that pharmacological inhibition of PI3Kγ with AS605240 suppressed the progression of joint inflammation and damage in rheumatoid arthritis induced in mice.

We have also investigated the involvement of PI3Kγ in the spontaneous nociception induced by trypsin. Our present data are in line with a series of previous studies indicating that nonspecific PI3K inhibition is able to attenuate pain behaviors [11,30,39]. Indeed, this is the first time that a selective inhibitor of PI3Kγ is evaluated in a spontaneous nociception model. The oral treatment with AS605240 at 1 mg/kg produced a strong inhibition of spontaneous nociception elicited by trypsin. Notably, when mice were treated with AS605240 by i.c.v route, the nociceptive behavior was virtually abolished. However, the i.t. injection caused only a small reduction of trypsin-induced licking behavior, as it was observed for the scratching behavior. Our findings are supported by previous studies evaluating the effects of different compounds through i.c.v. and i.t. routes in inflammatory and hyperalgesic models. For instance, Hajhashemi et al. [12] demonstrated that intraperitoneal (i.p.) and i.c.v. injections of maprotiline were able to significantly reduce carrageenan-evoked paw edema in rats, while i.t. treatment had no effect. The hypothesis raised by the authors is that maprotiline has central actions and it might alter the activity of descending neuronal pathways. In addition, Morgenweck et al. [24] have also reported that i.t. injection of peroxisome proliferator-activated receptor γ (PPARγ) agonists did not change carrageenan-induced edema or hyperalgesia in rats. Nevertheless, the i.c.v administration decreased paw edema, hyperalgesia and spinal c-Fos expression, likely indicating an improvement of descending inhibition, reduction of descending facilitation, or induction of spinal pain

transmission modulation. Similar mechanisms of pain processing modulation might well be implicated in the effects of AS605240.

It is well known that noxious stimulation induces expression of c-Fos, which is widely used as a pain-related marker [13].Then, immunohistochemistry was also performed, as done for the pruritus model, to verify the ability of the AS605240 in modulating central mechanisms of sensorial pain transmission. As expected, trypsin injection into the mouse paw induced an upregulation of c-Fos in the spinal cord, while PI3Kγ inhibition by oral administration of AS605240 resulted in a discrete reduction of c-Fos expression.

Recently, it has been demonstrated that injection of compounds such as capsaicin or formalin, promotes an increase in Akt phosphorylation [30,36]. In line with the literature data, trypsin injected into the mouse paw augmented the phospho-Akt immunoreactivity at the spinal level in our study. Interestingly, PI3Kγ inhibition with AS605240 significantly decreased the phospho-Akt immunolabeling.

In order to assess whether AS605240 had effect on nociception caused by other agent, the capsaicin model was also evaluated. Unlike it was reported in some previous studies [36,40], we showed herein that PI3K inhibition did not modify the pain-like behavior induced by capsaicin. The difference between our results and literature data might rely on the administration route and lack of selectivity to a specific isoform, once they injected PI3K pan-inhibitors intraplantarly. Nevertheless, our data suggests that PI3Kγ activation does not appear to be crucial for capsaicin-induced pain-like behavior. In fact, TRPV1 activation leads to the stimulation of a series of intracellular signaling pathways in addition to PI3K [40]. Taken together, the present findings suggest that PI3Kγ activation represents one of the important mechanisms responsible for trypsin-induced inflammation, nociception and pruritus, since the pharmacological blockage of

PI3Kγ resulted in decreasing the responses caused by this proteinase. Additionally, our data provide convincing evidence indicating that PI3Kγ is quietly relevant for PAR-2-mediated responses. Further studies are still necessary to better understand the central effects of the PI3Kγ inhibition. In conclusion, our data indicate that blockade of PI3Kγ might represent a valuable alternative for treating inflammatory and painful conditions as well as pruritus.

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Figures:

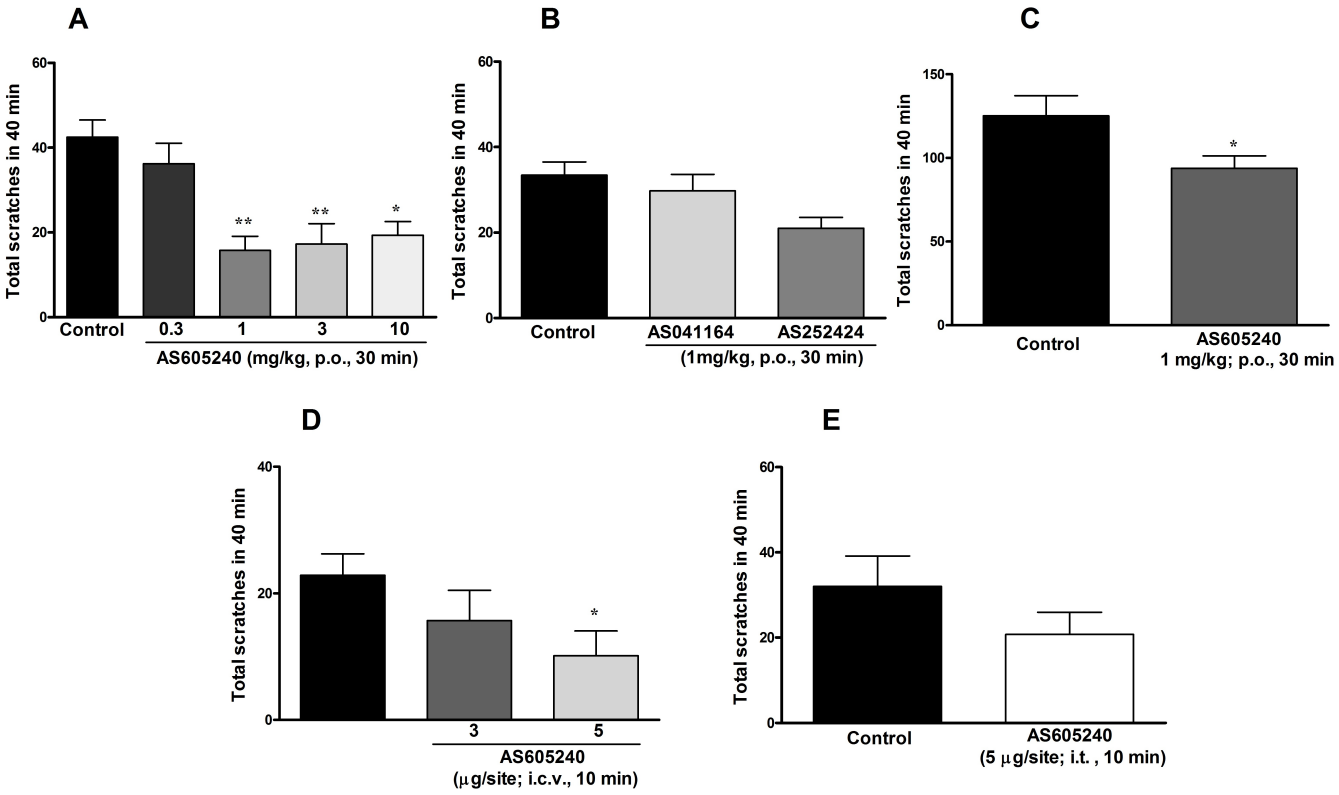

Figure 1- Effect of treatment with the selective PI3Kγ inhibitors on pruritus models. (A) AS605240 (0.3 to 10 mg/kg) administered p.o. 30 min before trypsin-induced scratching behavior. (B) AS041164 and AS252424 (both 1 mg/kg) given p.o. 30 min before trypsin-elicited scratching behavior. (C) AS605240 (1 mg/kg) administered p.o. 30 min before scratching behavior evoked by compound 48/80. (D) AS605240 administered i.c.v. (3 and 5 μg/site) 10 min before scratching behavior caused by trypsin. (E) Intrathecal injection of AS605240 (5 μg/site) 10 min before trypsin-induced pruritus. Each column represents the mean of 8 animals and the vertical lines show the S.E.M. *Denotes the significance levels in comparison to control values: *P<0.05; **P<0.01

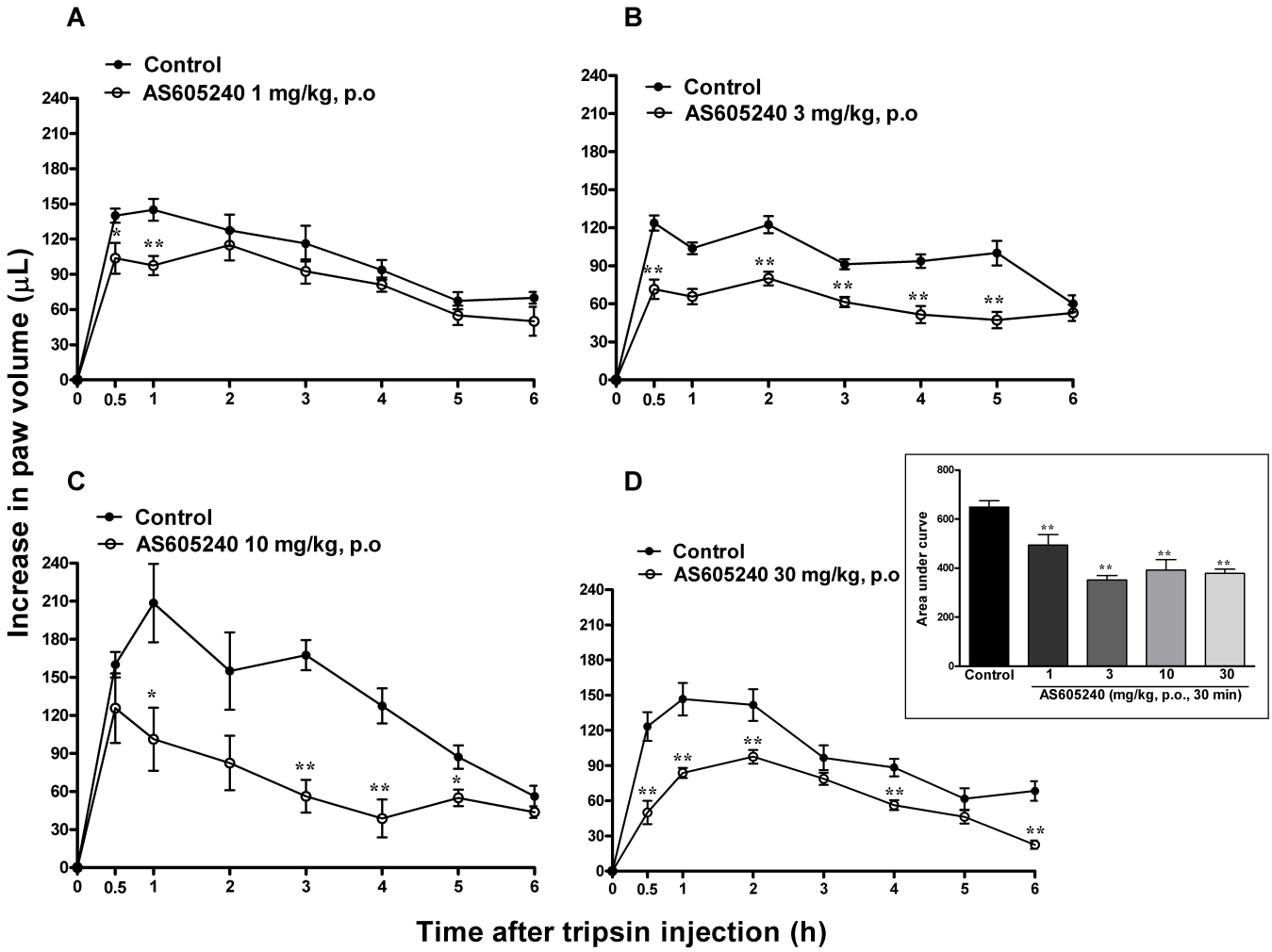

Figure 2 - Effect of treatment with the selective PI3Kγ inhibitor AS605240 (A) 1 mg/kg; p.o., (B) 3 mg/kg; p.o., (C) 10 mg/kg; p.o. and (D) 30 mg/kg; p.o., administered 30 min before, on the increase in hindpaw volume evoked by trypsin in mice. Each point on the curve represents the mean of 8 animals and the vertical lines show the S.E.M. Asterisks denote the significance levels in comparison to control values: *P<0.05; **P<0.01

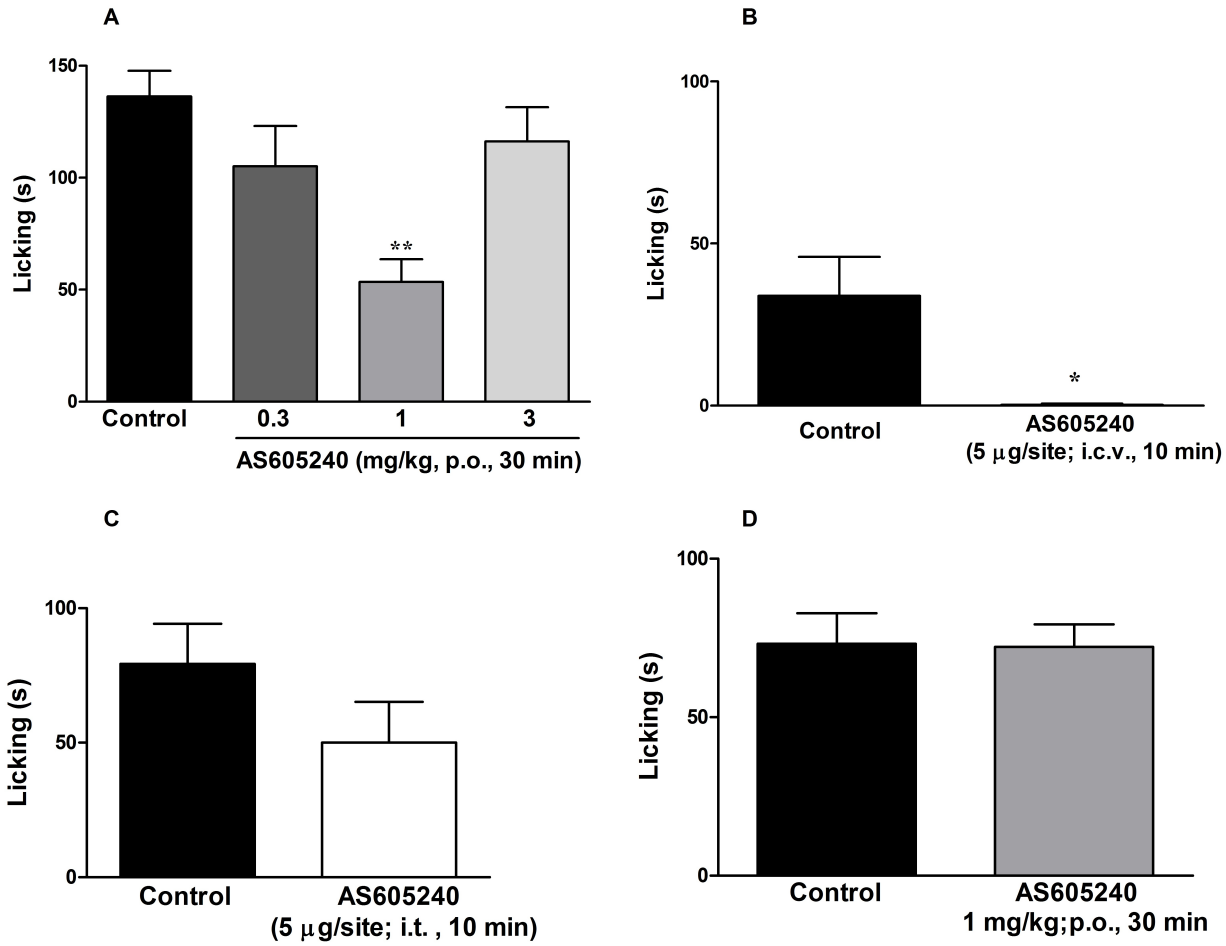

Figure 3 - Effect of treatment with the selective PI3Kγ inhibitor AS605240 on nociceptive models. (A) Orally given 30 min (0.3 to 3 mg/kg) before trypsin-elicited spontaneous nociception. (B) Injected i.c.v (5 µg/site) 10 min before trypsin-evoked nociception. (C) Given i.t. (5 µg/site) 10 min before the spontaneous nociception induced by trypsin. (D) Administered p.o. (1 mg/kg) 30 min before the nociceptive response caused by capsaicin. Each column represents the mean of 8 animals and the vertical lines show the S.E.M. *Denotes the significance levels in comparison to control values: *P<0.05; **P<0.01

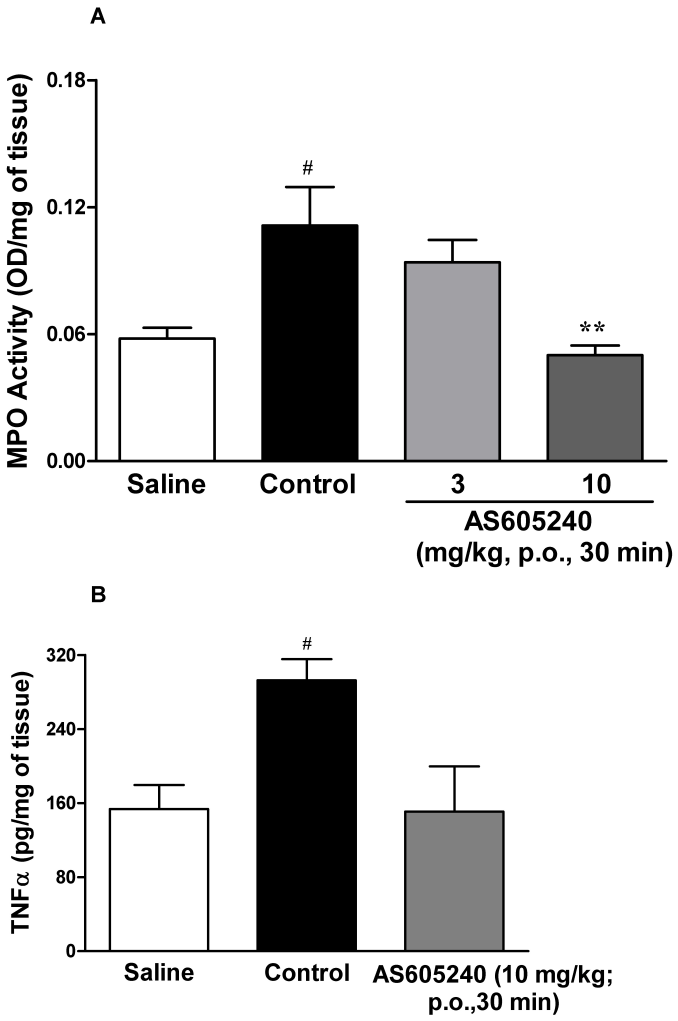

Figure 4- Effects of treatment with AS605240 (3 and 10 mg/kg), administered p.o. 30 min before, on inflammatory parameters. (A) MPO activity following trypsin injection into the mouse paw. (B) TNFα levels after trypsin injection into the mouse paw. Each column represents the mean of 8 animals and the vertical lines show the S.E.M. *Denotes significance in comparison to control values. $^{\#}$Denotes significance in comparison to saline-injected mice. **P<0.01 and $^{\#}$P<0.01

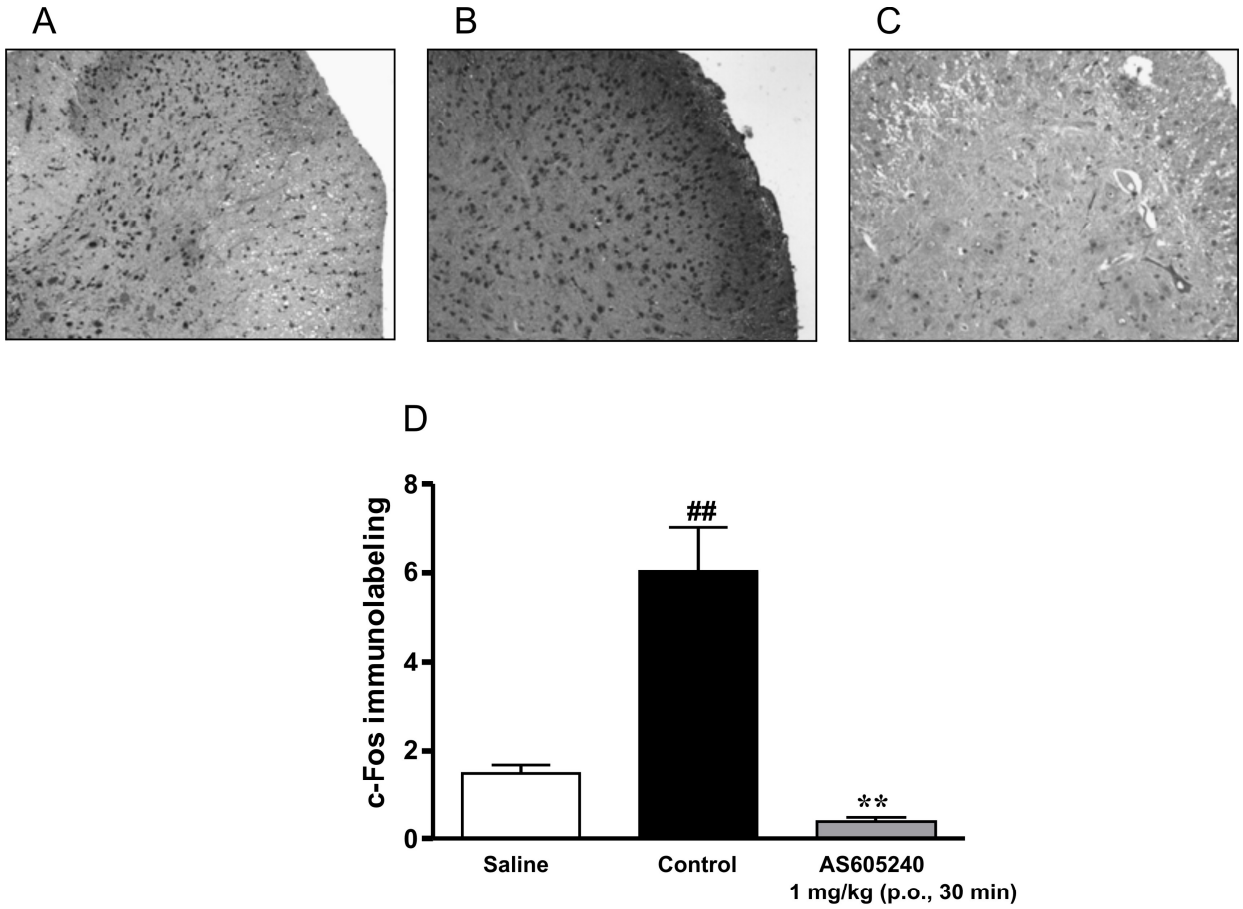

Figure 5 – Immunohistochemistry analysis for c-Fos after injection into the dorsum. (A) Saline-injected animals. (B) Trypsin-injected animals. (C) Mice treated with AS605240 (1 mg/kg) administered p.o. 30 min before injection of trypsin.Original magnification: 100x (D) Graphic representation of immunostaining for c-Fos. Each column represents the mean of 4 animals and the vertical lines show the S.E.M. *Denotes the significance levels in comparison to control values. #Denotes significance in comparison to saline-injected mice. **P<0.01 and ##P<0.01

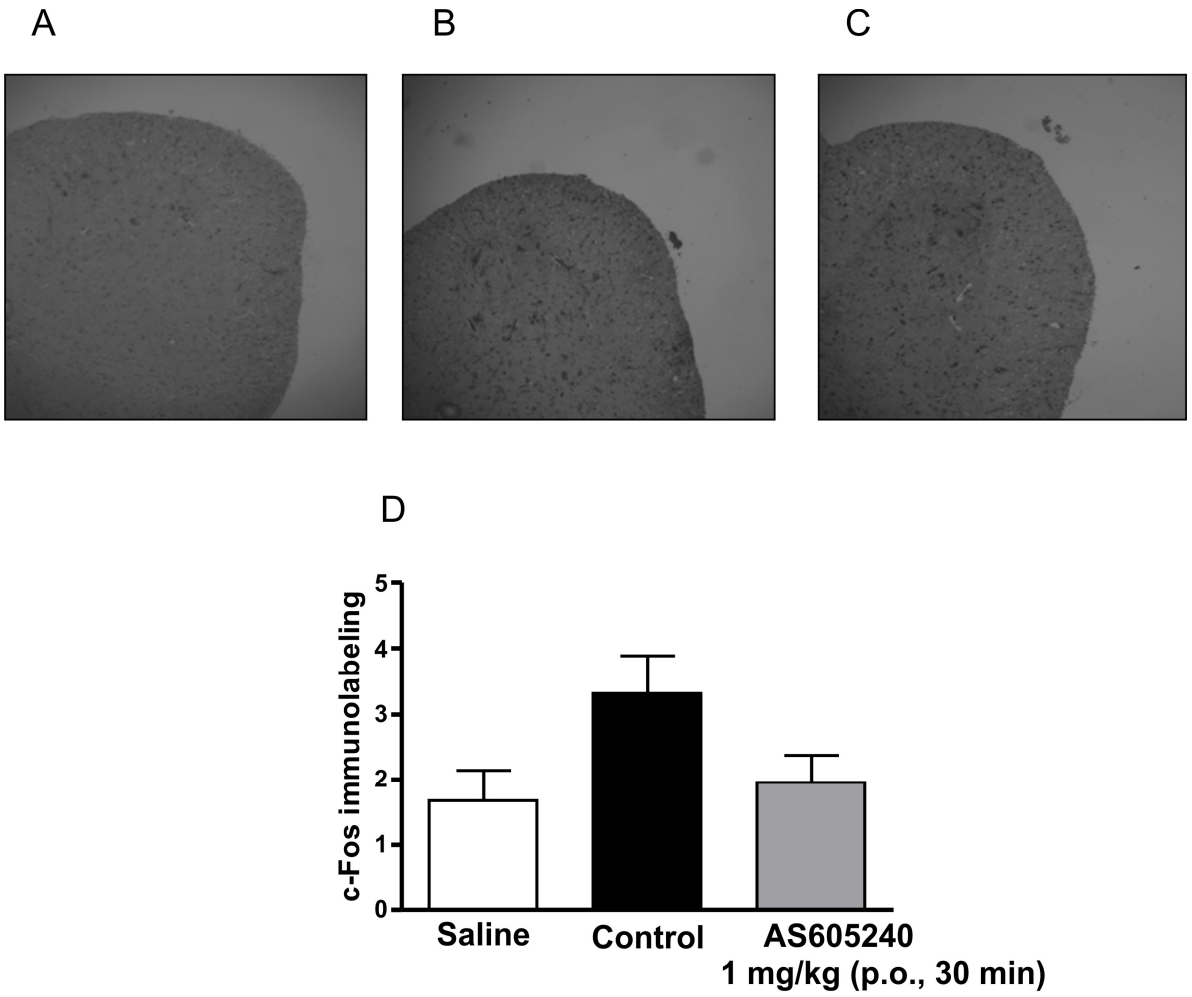

Figure 6 – Immunohistochemistry analysis for c-Fos after injection into the right hindpaw. (A) Saline-injected animals. (B) Trypsin-injected animals. (C) Mice treated with AS605240 (1 mg/kg) administered p.o. 30 min before trypsin injection. Original magnification: 100x (D) Graphic representation of immunostaining for c-Fos. Each column represents the mean of 4 animals and the vertical lines show the S.E.M.

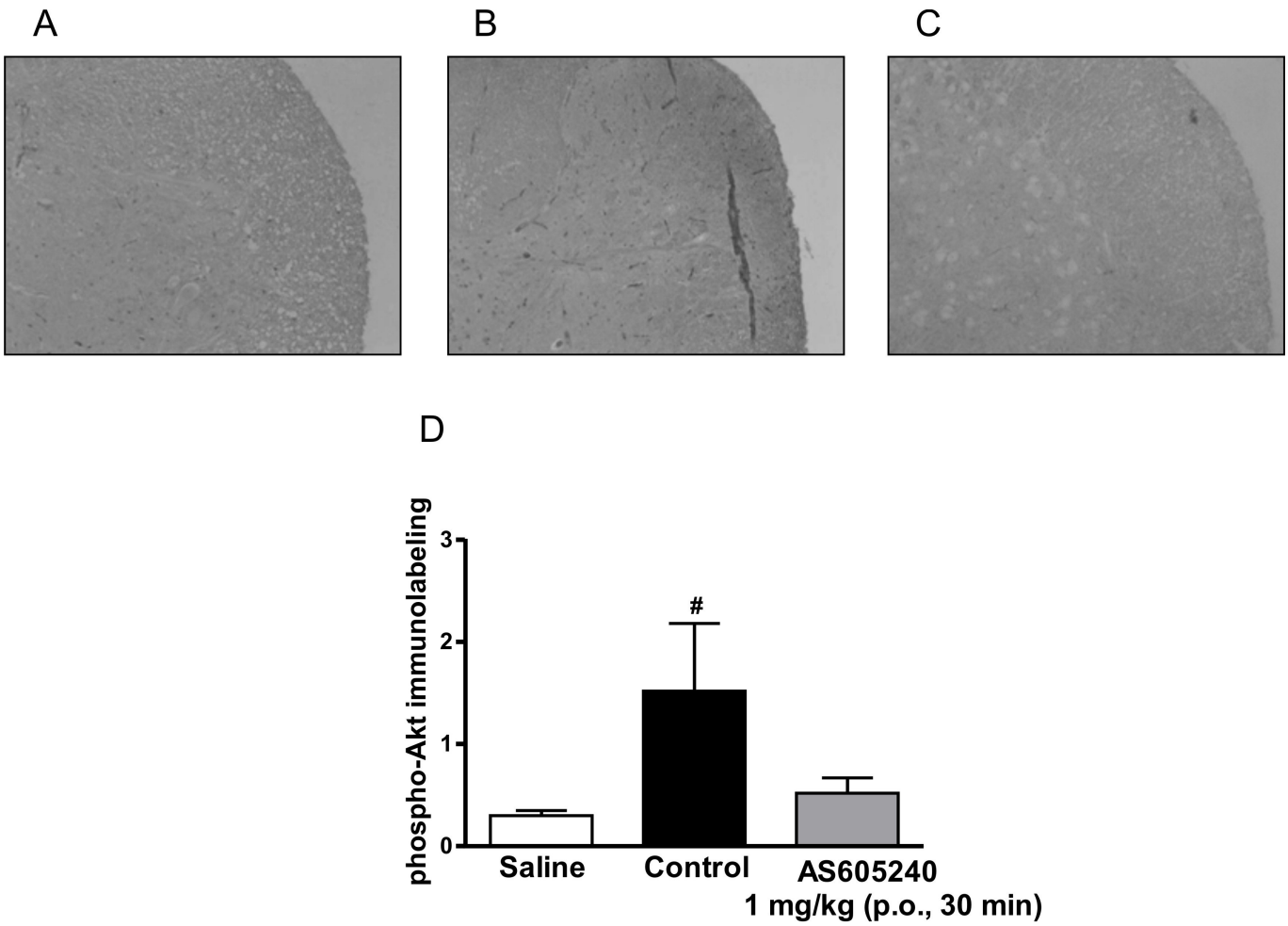

Figure 7 – Immunohistochemistry analysis for phospho-Akt after injection into the dorsum. (A) Saline-injected animals. (B) Trypsin-injected animals. (C) Mice treated with AS605240 (1 mg/kg) administered p.o. 30 min before trypsin injection. Original magnification: 100x (D) Graphic representation of immunostaining for phospho-Akt Each column represents the mean of 4 animals and the vertical lines show the S.E.M. #Denotes the significance in comparison to saline-injected mice. #P<0.05

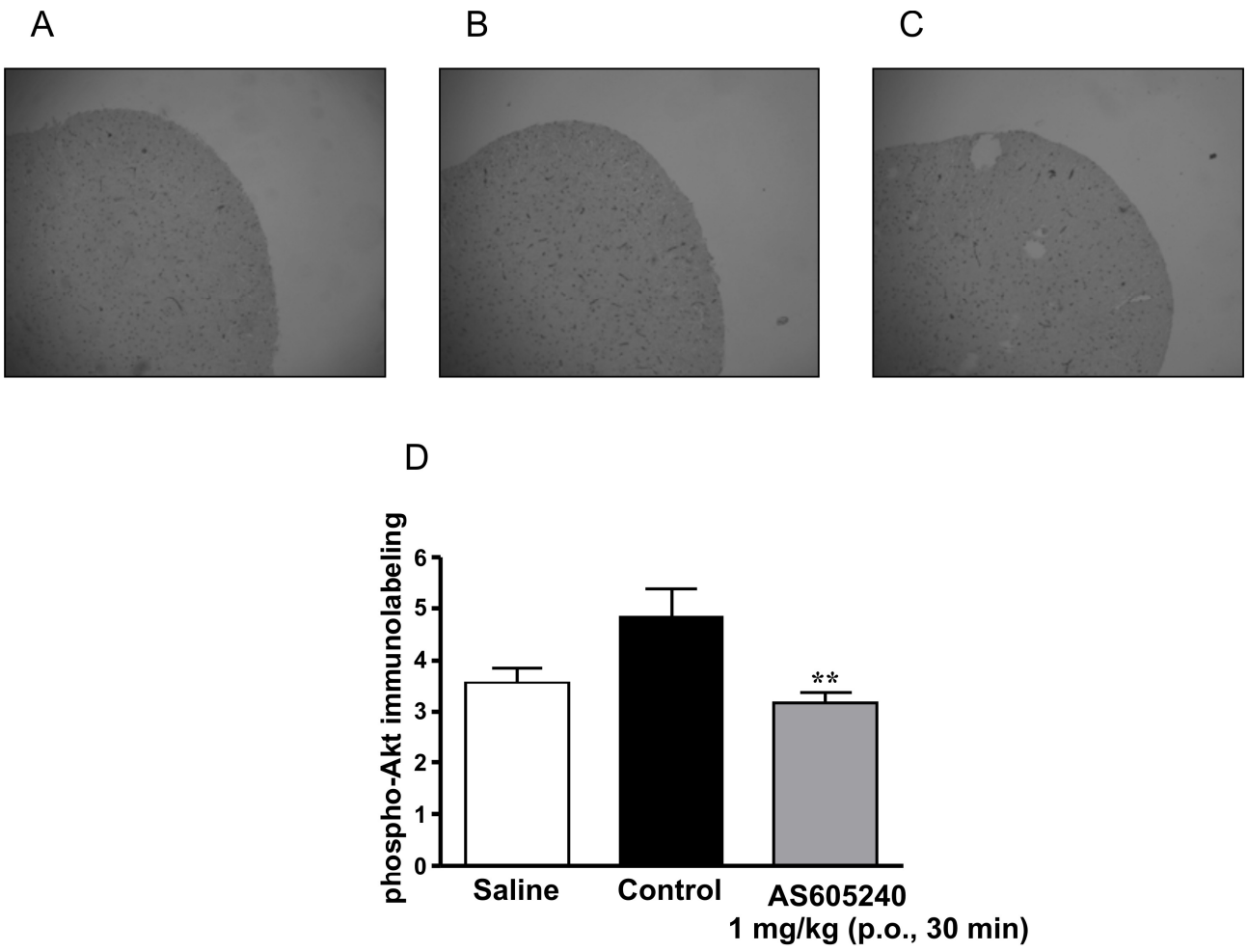

Figure 8 – Immunohistochemistry analysis for phospho-Akt after injection into the right hindpaw. (A) Saline-injected animals. (B) Trypsin-injected animals. (C) Mice treated with AS605240 (1 mg/kg) administered p.o. 30 min before injection of trypsin. Original magnification: 100x (D) Graphic representation of immunostaining for phospho-Akt. Each column represents the mean of 4 animals and the vertical lines show the S.E.M. *Denotes the significance levels in comparison to control values. **P<0.01

4. CONSIDERAÇÕES FINAIS

A descoberta de receptores especificamente ativados por proteases, os receptores PARs, introduziu o conceito de que estas proteínas não são apenas enzimas de degradação, mas também, importantes moléculas sinalizadoras (Cirino & Vergnolle, 2006). Sabe-se que as proteases são liberadas durante processos patológicos, incluindo câncer. Dentre as serino-proteases pode-se destacar a tripsina (Koshikawa et al., 1998; Sánchez-Hernández et al., 2008; Vergnolle et al., 2001a). Recentemente, tem sido descrito que a tripsina é capaz de induzir inflamação e nocicepção, estando associada também à dor causada por câncer em camundongos, principalmente pela ativação do receptor PAR-2 (Lam & Schmidt, 2010; Paszcuk et al., 2008). Além disso, a tripsina pode causar prurido, também pela ativação do receptor PAR-2 (Costa et al., 2008). Diversos estudos demonstram que as respostas nociceptivas e inflamatórias induzidas pela tripsina *in vivo* parecem depender da ativação direta de nociceptores, da intensa infiltração de neutrófilos e da liberação de diferentes mediadores da inflamação, incluindo bradicinina, prostanoides, neuropeptídeos, serotonina (5-HT) e histamina, além da ativação de receptores PAR-2 localizados nas fibras C, fibroblastos, queratinócitos ou mastócitos, o que leva à liberação de diferentes moléculas, gerando respostas pruriginosas (Vergnolle et al., 2001a; Vergnolle et al., 1999; Vergnolle et al., 2001b).

Embora o receptor PAR-2 esteja relacionado com uma série de condições, principalmente patológicas, ainda se sabe pouco ainda sobre as vias de sinalização ativadas por sua estimulação, especialmente durante alterações pruriginosas, inflamatórias e nociceptivas. Assim, a melhor identificação dessas vias de sinalização intracelular é de extrema importância para posteriormente poder utilizá-las como alvos terapêuticos para uma série de doenças.

A PI3Kγ é ativada via receptores acoplados à proteína G, sendo preferencialmente expressa nas células do sistema imune; porém, também pode ser encontrada no cérebro. Evidências sugerem, especialmente nos últimos 10 anos, que esta isoforma desempenha um papel crucial durante o processo inflamatório (Ghigo et al., 2010; Peng et al., 2010; Rückle et al., 2006). Isso se deve principalmente à capacidade de ativar e recrutar leucócitos durante a resposta inflamatória (Passos et al., 2010). Muitos estudos mostram que o aumento constitutivo da sinalização da via da PI3K pode ter efeito deletério nas células, levando ao descontrole da proliferação, migração facilitada e adesão independente de crescimento. Estes eventos favorecem a formação de tumores malignos, o desenvolvimento de inflamação crônica, diabetes, alergia, problemas cardiovasculares e doenças autoimunes (Gharbi et al., 2007; Ito et al., 2007 ; Marone et al., 2008). Ademais, trabalhos recentes têm descrito que a ativação da via da PI3K pode contribuir para o desenvolvimento de estados dolorosos agudos e crônicos (Guan et al., 2010; Pezet et al., 2008; Xu et al., 2007). No entanto, a ativação desta via em processos pruriginosos ainda não foi estudada. A inibição seletiva da PI3Kγ constitui um novo conceito para o controle de alterações presentes em doenças inflamatórias e autoimunes (Ferrandi et al., 2007; Rückle et al., 2006).

O presente trabalho investigou a participação da PI3Kγ nas respostas pruriginosas, inflamatórias e nociceptivas mediadas pela tripsina *in vivo*, através do emprego de técnicas farmacológicas, bioquímicas e de biologia molecular. Os nossos resultados mostraram pela primeira vez que a inibição da PI3Kγ, pela administração de inibidores seletivos desta isofroma por via oral, reduziu o comportamento de coçar induzido pela tripsina em camundongos. A mesma estratégia foi capaz de produzir apenas redução parcial do comportamento de coçar induzido pelo degranulador de mastócitos, composto 48/80, indicando uma menor participação da PI3Kγ no prurido

mediado por mastócitos. Quando o bloqueio farmacológico da PI3Kγ foi realizado por via intracerebroventricular (i.c.v.) ou intratecal (i.t.), o comportamento de coçar induzido pela tripsina também foi reduzido, em especial pela via i.c.v. Assim, nossos resultados apontam um papel mais importante para a ativação cerebral da PI3Kγ, do que espinhal, no prurido causado pela tripsina. Também foi possível observar que o tratamento por via oral com o inibidor de PI3Kγ AS605240 diminuiu a imunomarcação para c-Fos e fosfo-Akt na medula espinhal induzida pela injeção de tripsina no dorso de camundongos. Com base neste conjunto de resultados é possível sugerir que a ativação da PI3Kγ pode estar envolvida na transmissão pruriginosa, aumentando o conhecimento atual sobre os mecanismos implicados na transmissão da coceira.

No presente estudo, observou-se que a inibição da PI3Kγ utilizando o AS605240 por via oral, também produziu uma redução de parâmetros inflamatórios como a formação de edema de pata, infiltração de neutrófilos e os níveis de TNFα induzidos pela tripsina na pata, o que indica que a PI3Kγ também participa das respostas inflamatórias evocadas pela tripsina. Ainda é possível inferir que a modulação de parâmetros inflamatórios, tais como a migração celular, pode ser responsável, ao menos em parte, pelos efeitos anti-pruriginosos observados pela inibição seletiva da PI3Kγ.

De maneira interessante, quando o inibidor AS605240 foi administrado por via oral na dose de 1 mg/kg, houve uma diminuição considerável da nocicepção espontânea evocada pela tripsina. Notavelmente, a injeção i.c.v. do inibidor AS605240 praticamente aboliu o comportamento nociceptivo induzido pela tripsina, enquanto ocorreu apenas uma pequena redução com a administração por via i.t. Neste modelo, o tratamento oral com AS605240 também foi capaz de reduzir a

imunopositividade para c-Fos e fosfo-Akt na medula espinhal, após a injeção de tripsina na pata de camundongos. Por outro lado, no modelo de nocicepção espontânea causada por capsaicina, não se observou redução no comportamento dos animais tratados com o inibidor de PI3Kγ. Isso parece indicar uma ação mais seletiva para os efeitos causados pela tripsina e possivelmente pela ativação do receptor PAR-2, com relação à via da PI3Kγ.

Com base nos dados aqui apresentados é possível inferir que: (i) a ativação da PI3Kγ representa um dos mecanismos responsáveis pelas respostas inflamatórias, nociceptivas e, principalmente, pruriceptivas causadas pela tripsina; (ii) a inibição da PI3Kγ pode representar uma alternativa importante para o tratamento de processos inflamatórios, dolorosos e pruriginosos. Estas evidências permitem sugerir que inibidores seletivos e ativos por via oral, como o AS605240, podem representar alternativas interessantes para o tratamento da dor e da coceira associadas a doenças crônicas, especialmente naquelas onde os receptores PAR-2 estão diretamente implicados.

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ANEXO I

Documento de confirmação de submissão do manuscrito

From: Pain <painjournal@iasp-pain.org>
To: camposmmartha@yahoo.com; maria.campos@pucrs.br
Sent: Fri, December 10, 2010 2:26:20 PM
Subject: A manuscript number has been assigned

Journal: Pain
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ID: PAIN-D-10-7820
Format: Full-Length Article
Authors: Paula J Seadi Pereira; Lais F Lazarotto; Paulo C Leal ; Tiago G Lopes; Fernanda B Morrone ; Maria Martha Campos, Ph.D.

Dear Dr Campos,

Your submission entitled "Inhibition of phosphatidylinositol-3 kinase <gamma> reduces pruriceptive, inflammatory and nociceptive responses induced by trypsin in mice" has been assigned the following manuscript number: PAIN-D-10-7820.

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