UNIVERSIDADE ESTADUAL DE CAMPINAS
FACULDADE DE ODONTOLOGIA DE PIRACICABA

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Envolvimento das células microgliais na instalação e manutenção da hiperalgesia persistente induzida na pata de ratos

Tese de Doutorado apresentada à Faculdade de Odontologia de Piracicaba, da Universidade Estadual de Campinas, para obtenção do Título de Doutor em Odontologia, na Área de Fisiologia Oral

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Este exemplar corresponde à versão final da Tese defendida pela aluna Marília Bertoldo Urtado e orientada pela Profa. Dra. Juliana Trindade Clemente Napimoga.

Assinatura do Orientador

PIRACICABA
2012

FICHA CATALOGRÁFICA ELABORADA POR
MARILENE GIRELLO – CRB8/6159 - BIBLIOTECA DA
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Ur8e

Urtado, Marília Bertoldo, 1976-
Envolvimento das células microgliais na instalação e manutenção da hiperalgesia persistente induzida na pata de ratos / Marília Bertoldo Urtado. -- Piracicaba, SP : [s.n.], 2012.

Orientador: Juliana Trindade Clemente Napimoga.
Coorientador: Maria Cecília Ferraz de Arruda Veiga.
Tese (doutorado) - Universidade Estadual de Campinas, Faculdade de Odontologia de Piracicaba.

1. Minociclina. 2. Dor. 3. Neuroglia. I. Napimoga, Juliana Trindade Clemente. II. Veiga, Maria Cecília Ferraz de Arruda, 1953- III. Universidade Estadual de Campinas. Faculdade de Odontologia de Piracicaba. IV. Título.

Informações para a Biblioteca Digital

Título em Inglês: Involviment of microgliais cells in the installation and maintenance of persistent hyperalgesia induced in the paw of rats
Palavras-chave em Inglês:
Minocycline
Pain
Neuroglia
Área de concentração: Fisiologia Oral
Titulação: Doutor em Odontologia
Banca examinadora:
Maria Cecília Ferraz de Arruda Veiga
Maria José Alves da Rocha
Mariana Trevisani Arthuri Franco
Wander José da Silva
Míriam Elias Cavallini
Data da defesa: 28-02-2012
Programa de Pós-Graduação: Odontologia

UNIVERSIDADE ESTADUAL DE CAMPINAS
Faculdade de Odontologia de Piracicaba

A Comissão Julgadora dos trabalhos de Defesa de Tese de Doutorado, em sessão pública realizada em 28 de Fevereiro de 2012, considerou o candidato MARÍLIA BERTOLDO URTADO aprovado.

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Prof. Dr. WANDER JOSÉ DA SILVA

Profa. Dra. MÍRIAM ELIAS CAVALLINI

Dedico esse trabalho a minha mãe, Maria José, pelo amor que me sustentou em todos os momentos e por uma vida de dedicação e trabalho que possibilitou aos seus filhos a oportunidade de realizar sonhos e conquistas.

AGRADECIMENTOS

Agradeço primeiramente a **Deus** por possibilitar-me mais essa conquista.

À **Universidade Estadual de Campinas**, na pessoa do Magnífico Reitor **Prof. Dr. Fernando Ferreira Costa.**

À **Faculdade de Odontologia de Piracicaba da Universidade Estadual de Campinas**, na pessoa do seu Diretor, **Prof. Dr. Jacks Jorge Junior.**

À **Coordenadora dos Cursos de Pós-Graduação** da Faculdade de Odontologia de Piracicaba da Universidade Estadual de Campinas, **Profa. Dra. Renata Cunha Matheus Rodrigues Garcia.**

À **Coordenadora do Programa de Pós-Graduação em Odontologia** da Faculdade de Odontologia de Piracicaba, **Profa. Dra. Cinthia Pereira Machado Tabchoury.**

À **Profa. Dra. Renata de Oliveira Mattos Graner** do Programa de Pós-Graduação em Biologia Buco-Dental, Área de Microbiologia e Imunologia, da Faculdade de Odontologia da Piracicaba, Universidade Estadual de Campinas, pela permissão de uso do laboratório da área.

Às minhas orientadoras **Profa. Dra. Maria Cecília Ferraz de Arruda Veiga** e **Profa. Dra. Juliana Trindade Clemente Napimoga** pela excelência profissional, amizade e dedicação sempre indicando a direção a ser tomada em todos os momentos, pelo exemplo de determinação e competência. Obrigada por acreditarem nos meus projetos, lapidar meus conhecimentos contribuindo para minha evolução profissional e pessoal.

Ao **Prof. Dr. Wander José da Silva,** do Programa de Pós Graduação em Clínica Odontológica, Área Prótese Dental, da Faculdade de Odontologia da

Piracicaba, Universidade Estadual de Campinas, pelos ensinamentos em microscopia confocal.

À **Profa. Dra. Célia Mariza Rizatti Barbosa**, do Programa de Pós Graduação em Clínica Odontológica, Área Prótese Dental, da Faculdade de Odontologia da Piracicaba, Universidade Estadual de Campinas, pela oportunidade de trabalho na clínica e incentivos constantes.

Ao **Prof. Dr. Christiano Bertoldo Urtado,** da Faculdade de Ciências Medicas da Universidade Estadual de Campinas, por me acompanhar desde e início contribuindo com meu aprimoramento. Obrigada pelo apoio, paciência e ensinamentos.

À **Profa. Dra. Érica Harth Chú**, do Laboratório de Micro e Imunologia da Faculadade de Odontologia da Piracicaba, Universidade Estadual de Campinas, por todos os ensinamentos em biologia molecular.

À **Profa. Dra. Míriam Elias Cavallini**, do Laboratório de Farmacologia da Faculdade de Odontologia da Piracicaba, Universidade Estadual de Campinas pelo apoio e incentivo.

Aos professores doutores**, Ana Paula de Souza Pardo, Christie Leite Passini, Felippe Bevilacqua Prado, Franco Arsati, Gilson Cesar Nobre Franco, Maria José Alves Rocha, Karina Cogo, Mariana Travisani Arthuri Franco,** que participaram dessa fase da minha vida contribuindo para a conclusão desse trabalho

A todos do **Programa de Pós- Graduação em Odontologia** da Faculdade de Odontologia de Piracicaba, Universidade Estadual de Campinas, pela atenção e ensinamentos.

Aos meus pais **Maria José Bertoldo Urtado e Carlos Roberto Urtado**, pelo amor e contínuo apoio em todos estes anos, ensinando-me, principalmente, a importância da construção dos valores éticos e morais.

Ao meu irmão **Christiano Bertoldo Urtado** pelo amor e incentivo**.** Sempre pronto, sempre dedicado, agradeço a presença e apoio constantes.

Ao meu marido **André Bonetti**, por contribuir, efetivamente, para lapidação da minha alma e evolução do meu espírito.

Aos meus filhos, **Nícollas Bertoldo Bonetti** e **Filippo Bertoldo Bonetti** por serem os maiores incentivadores da minha vida e por me ensinarem que tudo é possível.

À amiga **Nádia Cristina Fávaro Moreira** pela presença e apoio na reta final dessa minha trajetória.

Agradeço à **Karla Helena Torres Chávez, Gustavo Alberto Obando-Pereda, Christina Macedo, André Vieira,** que de uma forma ou de outra, contribuíram para realização deste trabalho, gostaria de expressar minha gratidão.

Ao senhor **Carlos Alberto Feliciano**, agradeço pela ajuda no dia-a-dia do laboratório.

Às Sr. **Maria Elisa dos Santos e Eliana Melo Franco** secretárias do Programa de Pós-Graduação em Odontologia e **Sra. Eliete Rigueto,** secretária do departamento de Ciências Fisiológicas da Faculdade de Odontologia de Piracicaba, obrigada pela excelência profissional, competência e por estarem sempre prontas a ajudar;

À Sras. **Érica Alessandra Pinho Sinhoreti e Rachel Q. Marcondes Cesar Sacchi** secretárias e assessora, respectivamente, da coordenadoria Geral dos

Programas de Pós-Graduação da Faculdade de Odontologia de Piracicaba pela competência e por estar sempre prontas a ajudar.

Ao **Conselho Nacional de Desenvolvimento Científico e Tecnológico – CNPq,** pela concessão da bolsa de estudo.

.

“A razão cardeal de toda a superioridade humana é sem dúvida a vontade. O poder nasce do querer. Sempre que o homem aplicar a perseverante energia de sua alma a um fim, ele vencerá obstáculos e se não atingir o alvo, pelo menos fará coisas admiráveis”

José de Alencar

RESUMO

A dor, até recentemente, entendida como sendo produto apenas da atividade neuronal. Hoje, apesar deste modelo neural ser aceito, as células gliais vem ganhando a atenção de pesquisadores uma vez que parecem estar ligadas a indução e manutenção da dor. Diante disso, o objetivo desse trabalho foi investigar papel das células microgliais e da proteína cinase ativada por mitogenos p38 (p38 MAPK) na hiperalgesia inflamatória aguda e persistente, induzida pela administração de Prostaglandina E_2 (PGE_2) na pata de ratos. A injeção intraplantar de PGE_2 induz um quadro de hiperalgesia, avaliada pelo teste Randall & Selito, que cessa completamente em 24 horas (período de indução da hiperalgesia). Entretanto, injeções intraplantares diárias de PGE_2 por 14 dias induzem uma hiperalgesia que persiste após o término do tratamento (período de manutenção da hiperalgesia). Os resultados deste estudo demonstraram que o pré-tratamento com injeção intratecal de Minociclina, um inibidor de atividade microglial, foi capaz de impedir a hiperalgesia mecânica aguda, mas não modificou a hiperalgesia mecânica persistente induzida pela PGE_2 na pata. A Minociclina também foi capaz reduzir os níveis CD11b, um marcador da ativação microglial, aumentado na medula espinhal coletada 48h após o tratamento com PGE_2. Níveis aumentados da p38 MAPK fosforilada (P-p38) foram observados na medula espinhal coletada 3h após injeção intraplantar de PGE_2. Imunofluorescência com marcação para microglia (CD11b) e para astrócito (GFAP) demonstrou co-localização entre P-p38 e células microgliais espinhais mas não com astrócitos Estes resultados sugerem que a hiperalgesia induzida pela PGE_2 na pata de ratos é mediada pela ativação das células microgliais espinhais e que a fosforilação da p38 MAPK age como um sinalizador intracelular para ativação destas células.

Palavras-chave: CD11b, hiperalgesia, microglia, minociclina, p38 MAPK, Prostaglandina E_2.

ABSTRACT

Pain conditions are traditionally viewed as being mediated only by neurons. However, it has been shown that spinal cord glial cells (microglia and astroglia) are very important to pain facilitation. The present work examined the role of microglial cells and p38 mitogen-activated protein kinase (MAPK), in the Prostaglandin E_2 (PGE_2)-induced mechanical paw hyperalgesia test. A single intraplantar injection of PGE_2 induced acute mechanical hyperalgesia 3h after its injection, measured by Randall-Sellito test. The acute hyperalgesia were completely solved 24h after PGE_2 injection (hyperalgesia induction period). Otherwise, rats treated with PGE_2 during fourteen days presented, after the disruption of the PGE_2 treatment, a persistent hyperalgesia (hyperalgesia maintenance period). The intrathecal administration of Minocycline, a microglial cell activation inhibitor, abolished PGE_2-induced acute paw hyperalgesia. However, intrathecal administration of Minocycline doesn´t inhibits PGE_2-induce persistent paw hyperalgesia. Minocycline significantly reduced the lumbar dorsal cord CD11b mRNA levels, a microglial activation marker that is increased in the PGE_2-induced acute hyperalgesia. Intraplantar injection of PGE_2 significantly increased the phosphorylated spinal microglial p38 MAPK 3 hours later. To confirm these results, immunocytochemisty double-staining with markers for microglia and astrocytes shown co-localization between phosphorylated p38 MAPK-immunoreactive cells and microglia, but not with other glia cells. Taken together, these results indicate that PGE_2-induced paw hyperalgesia is mediated by spinal microglial cells and phosphorylated spinal microglial p38 MAPK can be the upstream signal for spinal microglia cells activation.

Keywords: CD11b, hyperalgesia, microglia, minociclina, p38 MAPK, Prostaglandin E_2

SUMÁRIO

INTRODUÇÃO

A transmissão da dor a partir de estímulos periféricos para áreas ascendentes do cérebro é um processo dinâmico. A informação de dor é transmitida dos aferentes primários para o neurônio de transmissão via liberação de neuropeptídios (substancia P) e aminoácidos excitatórios (EAAs) como o glutamato, que atuam em receptores específicos (NK1, AMPA e NMDA) localizados no neurônio de projeção (Watkins *et al*., 2001). A partir daí a informação sensorial de dor é transmitida para os centros encefálicos superiores até chegar ao córtex para ser interpretada. Embora esse modelo de transmissão da informação de dor envolvendo apenas neurônios seja aceitável, várias evidencias sustentam a hipótese de que as células gliais participam efetivamente no processamento da dor (Watkins *et al*., 2001).

Até recentemente as células gliais (astrócitos e microglia) eram consideradas células de suporte físico e metabólico para os neurônios. No entanto, estudos vêm demonstrando que esse tipo de célula pode contribuir com os mecanismos de indução e manutenção dor (Araque *et. al.,* 1999; Walkins *et al*., 2001; Milligan *et al*., 2001; Kim *et al*., 2002; Jin *et al.,* 2003; Raghavendra *et al*., 2003; Ledeboer *et al*., 2005; Ji *et al*.,2007; Suter *et al*., 2007; Li *et al.,* 2010). Foi demonstrado que as respostas nociceptivas aumentadas provocadas por lesão neural periférica ou inflamação estavam relacionadas com ativação das células gliais na medula espinhal (Garrison *et al.,* 1991; Garrison *et al*., 1994) e que a interrupção da ativação glial bloqueia essa resposta (Meller *et al*., 1994). Complementando estes dados, evidencias anatômicas e farmacológicas demonstraram de que a microglia ativada está relacionada com a iniciação/indução dos processos de dor exagerada e que os astrócitos são cruciais para a manutenção dessa dor exagerada (Raghavendra *et al*., 2003)

A microglia, célula fagocitária responsável pela imunidade inata do sistema nervoso central (SNC) (Rezaie *et al*., 2002), representa aproximadamente 5-12% das células deste sistema e é heterogeneamente distribuída (Lawson *et al*., 1990). Ela se origina da migração dos monócitos

derivados da medula marrom durante o período pré-natal (Nakajima & Kohsaka, 2001).

Microglia quiescente (quiescere, do latim: tornar-se quieto; não causar problemas ou sintomas) não é uma célula passiva e sim uma célula que monitora o tempo todo o ambiente em que vive por meio de seus prolongamentos ramificados (Nimmerjahn *et al*., 2005; Raivich, 2005).

A ativação da microglia pode ocorrer em resposta a alterações no SNC tais como, trauma, isquemia, tumores, neurodegeneração, processos inflamatórios e infecciosos (Tsuda *et al*., 2005). Uma vez ativadas essas células podem apresentar alterações morfológicas: de ramificadas para amebóides (Eriksson *et al*., 1993), aumento da expressão de marcadores microgliais, tais como o CD11b e aumento do número de células pela proliferação celular (Eriksson *et al*., 1993; Liu *et al*., 1995; Coyle, 1998).

A proliferação celular, na medula espinhal, é uma importante característica da ativação das células gliais uma vez que essa célula raramente se divide na medula espinhal intacta (Horner *et al*., 2000). Esse aumento do número celular é experimentalmente investigada usando bromodeoxyuridine (BrdU) um nucleotídeo que pode ser incorporado ao DNA de células em duplicação e reconhecido por anticorpos analisados por imunohistoquímica (Suter *et al.,* 2007) . Em diversos modelos de dor tais como lesão neural periférica (Graeber *et al*., 1988), transecção do nevo ciático (Liu *et al*., 2000), constrição do nervo ciático (Echeverry *et al*., 2008) e ligadura parcial do nervo ciático (Narita *et al*., 2006) a proliferação microglial pode ser observada. O mecanismo pelo qual a proliferação das células microgliais contribui para as respostas aumentadas de dor ainda não é bem esclarecido, mas acredita-se que resulte do aumento da produção de mediadores inflamatórios, levando a dor anormal.

Sem dúvida, a característica de ativação microglial mais estudada é o aumento da expressão de marcadores microgliais como o receptor de complemento 3 (CR3 ou CD11b, reconhecido pelo anti corpo OX42). O aumento da expressão de OX42 imunorreativo na medula espinhal, já foi demonstrado em vários modelos de dor inflamatória que não envolvem lesão

do nervo, tais como os que usam carragenina (Hua *et al*., 2005), Zymozan (Clark *et al*., 2007; Sweitzer *et al*., 1999), Adjuvant de Freund (Raghavendra *et al*., 2004) e formalina (Sweitzer *et al*., 1999; Fu *et al.,* 1999; Fu *et al*., 2000; Aumeerally *et al*., 2004;)

Tem sido demonstrado que as células microgliais espinhais podem ser ativadas por diversos mediadores tais como a substância P (Mariott & Wilkin, 1993), aminoácidos excitatórios - EAAs (Takamura *et. al*., 1996), ATP (Hide *et al*., 2000; Tsuda *et al*., 2003),liberados pelos terminais centrais dos neurônios sensoriais; assim como pelo óxido nítrico - ON (Mollace *et al*.,1998), prostaglandinas (Bezzi *et al*., 1998) e fractalkine (Maciejewski-Lenoir, D. *et al.,* 1999; Chapman, G. *et al*., 2000), liberados pelo neurônio de projeção.

A estimulação das células microgliais, pode aumentar a resposta dolorosa aumentando a produção do fator de crescimento derivado do cérebro (BDNF) (Coull *et al.,* 2005) inibindo a ativação do GABA (Coull *et al*., 2005); ativando receptores P2X7 através da liberação de ATP (Donnelly-Roberts *et al*., 2008; Inoue, 2007); e/ou aumentando a liberação de citocinas IL-1b,IL-6, IL-10, TNF no corno dorsal da medula espinhal, o que parece estar diretamente envolvido com o desenvolvimento dos estados exagerados de dor, uma vez que essas citocinas exercem um importante sinal de feedback autócrino além de mediar a interação neurônio-glia (Winkelstein *et al*., 2001, Verge *et al*., 2004).

Um proeminente envolvimento das proteinas kinases ativadas por mitógenos (MAPKs) vem ganhando destaque. Evidências sugerem que as MAPKs, incluindo a kinase regulada por sinal extracelular (ERK), p38 e a kinase c-Jun N-Terminal (JNK) desempenham um importante papel na indução da dor. Das 3 principais MAPKs, a p38 e a ERK são ativadas na microglia espinhal em condições de injúria e a ativação dessas 2 MAPKs parece ser importante para a sinalização microglial (Ji & Suter, 2007). Neste sentido, estudos demonstram a ativação pela fosforilação da p38 na microglia espinhal em diferentes modelos de dor neuropática e inflamatória (Kim *et al*., 2002; Svensson *et al*., 2003; Hua *et al*., 2005; Svensson *et al*., 2005; Hains & Waxman, 2006; Ji & Suter, 2007; Xu *et al*., 2007a; Xu *et al*., 2007b). Este

membro da família MAPK é normalmente ativado por estresse, calor, luz ultravioleta, isquemia e citocinas pró-inflamatórias (Widmann *et al.,* 1999). O envolvimento da p38 na ativação microglial faz das MAPKs alvos promissores no gerenciamento da dor (Jin *et al*., 2003, Stevensson, 2005).

Outro aspecto a ser abordado em relação ao estudo da atividade microglial no processamento da dor é o uso da Minociclina. A minociclina, substancia lipoproteica que possui habilidade de cruzar a barreira hematoencefálica (Aronson, 1998) é uma tetraciclina semi-sintética de segunda geração, que vem nos últimos anos chamando a atenção de pesquisados pelo seu efeito antiinflamatório que é totalmente distinto do seu poder antibiótico (Tikka *et al*., 2001; Tikka *& Koistinaho*, 2001). Estudos vêm demostrando o efeito da minociclina na diminuição da ativação da micróglia avaliada pela imunohistoquímica para CD11b ou outros marcadores microgliais em diversos modelos de dor inflamatória e neuropática (Yrjanheikki *et al*., 1998; Yrjanheikki *et al.,* 1999; Tikka & Koistinaho, 2001; Tikka *et al*., 2001).

Sendo a minociclina, considerada um inibidor da ativação microglial sem afetar diretamente neurônios ou astrócitos (Tikka *et al*., 2001; Raghavendra *et al*., 2003), seu efeito tem sido demonstrado in vitro (Yenari *et al* . , 2006) e em modelos experimentais agudos e crônicos (Tikka *et al*., 2001; Wu *et al*., 2005). Recentemente, tem sido demonstrado esse efeito anti-hiperalgésico e anti-alodínico em modelo de artrite, transecção do nervo espinhal e inflamação do ciático. (Raghavendra *et al*., 2003; Ledeboer *et al.,* 2005).

Considerando que a ativação microglial parece ser uma etapa importante para a indução e manutenção das respostas exageradas de dor, estudos relacionando ativação glial com modelos experimentais de indução e manutenção da hipernocicepção tornam-se relevantes para a pesquisa sobre a etiologia da cronificação da dor. Com base no exposto o presente estudo teve como principal objetivo investigar a participação das células microgliais espinhais na indução e manutenção da hipernocicepção induzida pela Prostaglandina E_2 (PGE_2) na pata de ratos.

Capítulo 1

PGE_2-induced peripheral hyperalgesia evokes spinal microglia activation

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Abstract

Activated microglia cells in spinal cord play a significant role in enhanced pain and might be a key for the development of exaggerated pain states of diverse etiologies. In the present study we report that intrathecal administration of Minocycline, a microglial cell activation inhibitor, abolished low mechanical threshold in acute hyperalgesia, evoked by Prostaglandin E_2 (PGE_2) injection, in the model of hyperalgesia induction, measured by Randall & Selito test. However, intrathecal administration of Minocycline doesn´t inhibits PGE_2-induce persistent hyperalgesia, but significantly reduced the lumbar dorsal cord CD11b mRNA levels, a microglial activation marker that is increased in the acute hyperalgesia induced by PGE_2 injection into rat´s paw. These results suggest that PGE_2-induced hyperalgesia can be mediated, at least in part, by central mechanisms involving microglial cells. The data highlight an important role of microglial activation in the development of enhanced pain states.

Keywords: CD11b, hyperalgesia, microglia, minocycline, pain, prostaglandin E_2

Introduction

It has been well established that release of arachidonic acid/cylco-oxygenase products and sympathomimetic amines, secondary to a cascade of cytokines released by defense cells responding to foreign stimuli, results in the activation of second messenger pathways (cAMP, Proteinkinase A and proteinkinase C) responsible for lowering the nociceptor threshold and increasing neuronal membrane excitability [5,36] -state called hyperalgesia. Moreover, frequent periods of nociceptors sensitization have been related to the development a persistent pain episode considering as a common source of chronic pain.

Despite many pain states are believed to reflect a significantly enhanced excitability of spinal pain processing neuronal circuits, it has been suggested that induction and maintenance of hyperalgesia also involves activation of spinal non-neuronal cells such as microglial cells [21,40]. In this sense, microglia in the spinal cord have been recognized as potential active participants in the initiation and maintenance of pain facilitation induced by inflammation and damage to peripheral tissues, peripheral nerves and spinal nerves [6, 40, 41]. Underneath stimulation by foreign antigens or in response to neuronal “stress” signals, microglia switch to a hyperactive phenotype, and may release inflammatory mediators that enhance the firing of nociceptive neurons, thus contributing to neuronal sensitization and behavioral hypersensitivity [15, 41].

Although the role of the spinal microglia cells in the initiation and maintenance of pain states has been reported [6, 40, 41], the spinal microglia cells activation in the Prostaglandin E_2 (PGE_2)-induced paw hyperalgesia has not been investigated. Considering that nociceptive behavioral responses elicited by PGE_2 injection in paw represent a valid and reliable model of induction and maintenance hyperalgesia [8], the aim of this study was to investigate the role of spinal microglia cells on PGE_2-induced paw hyperalgesia.

Material and methods

Animals: This study was carried out with adult male Wistar rats (200-300g) maintained in a temperature-controlled room (23° ± 1°C) with a 12-hour light–dark cycle. All experiments were conducted in accordance to the International Association for the Study of Pain guidelines on using laboratory animals for investigations of experimental pain in conscious animals [45]. All animals experimental procedures and protocols were approved by the Committee on Animal Research of the University of Campinas (protocol number 1574-1). The animals suffering and number per group were kept at a minimum (4-6 animals per group).

Experimental Design: Rats were treated with daily intraplantar (i.pl.) injection of PGE_2 (100ng/50µL) or saline (50µL) during fourteen days (induction period) [8,38]. The mechanical nociceptive threshold was assessed before (baseline) and 3 hours after each i.pl. injection of PGE_2 during the induction period and during the 4 days following the discontinuation of PGE_2 treatment (maintenance period). In order to avoid the local release of prostaglandins triggered by the trauma of i.pl. injection, all animals were treated with Indomethacin (2mg/Kg, intraperitoneal) 30 min. before the i.pl. injection [8]. To evaluate the role of spinal microglia cells on the induction and maintenance of PGE_2-induced paw hyperalgesia, animals were treated with intrathecal (i.t.) injection of minocycline (Mino) (100µg/10µL) [16], a microglial inhibitor, during 3 days before start the i.pl. PGE_2 injections (induction period) and during 3 days after disruption of treatment with PGE_2 (maintenance period). In order to confirm de activation of spinal microglia cells, after behavioral nociception test animals were killed and the lumbar spinal cord segments L5-6 were collected for assessed CD11b mRNA levels (microglial activation marker). The drugs used in this study were obtained from Sigma-Aldrich, MO, USA and were dissolved in sterile saline.

Intraplantar Injection: PGE_2 or its vehicle were subcutaneously injected in the dorsum of the rat's hind paw by tenting the skin and puncturing it with a 30-gauge needle prior to injecting the test agent, as previously described [25]. The

needle was connected to a catheter of polyethylene and also to a Hamilton syringe (50µL). The animals were briefly restrained and the total volume administered in the paw was 50µL.

Intrathecal injection: The intrathecal injections of Minocycline were performed under light halothane anesthesia (1/3 O_2 to 2/3 N_2O and halothane at 5% and 1.5%, respectively). The dorsal fur of each rat was shaved, the spinal column was arched, and a 26-gauge needle was directly inserted into the subarachnoid space, between the L4 and L5 vertebrae [22]. Correct intrathecal positioning of the needle tip was confirmed by manifestation of a characteristic tail flick response. The entire injection procedure, from the induction of anesthesia until recovery of consciousness, took ≈ 4 min.

Mechanical paw-withdrawal nociceptive threshold test: Testing sessions took place during light phase (between 09:00 A.M. and 5:00 P.M.) in a quiet room maintained at 23°C [29]. The Randall–Selitto nociceptive paw-withdrawal flexion reflex test [28] was performed using an Ugo-Basile analgesymeter (Stoelting, Chicago, IL, USA), which applies a linearly increasing mechanical force to the dorsum of the rat's hind paw [25]. The nociceptive threshold was define as the force in grams, with which the rat withdrew its paw. The basal paw-withdrawal threshold was defined as the mean of three measures performed at 5-min intervals before the first i.pl. injection of PGE_2. The variation (Δ) of mechanical hyperalgesia expressed on the graphs were calculated by subtracting the mean of three consecutive measurements after the treatment evaluated (test) from the basal threshold (Δ of mechanical hyperalgesia = basal – test).

RNA isolation and cDNA synthesis: Total RNA from lumbar dorsal spinal cord tissue was extracted 3h and 48h after i.pl. PGE_2 injection, using Trizol Reagent (Invitrogen life technologies, Carlsbad, CA) according to the manufacturer's instructions. Samples were treated with DNase to remove contaminating DNA using the Ambion DNA-free Kit (Ambion, Austin, TX). Concentration and purity of the RNA were determined by measuring the absorbance at 260 and 280nm

in a spectrophotometer. Total RNA was reverse transcribed into cDNA using Hight-Capacity cDNA Reverse Transcription Kits (Applied Biosystems). First-strand cDNA was synthesized using 2.0µg of total RNA, 2.0µg Buffer, 0.8µg 25X dNTP Mix (100mM), 2.0µL MulScribe Reverse Transcriptase, Nuclease-free H_2O in a total volume of 20µL. The reaction was carried out at 25℃ for 50 min, 37℃ for 120 min and terminated by deactivation of the enzyme at 85℃ for 5 min. Control reactions lacking either reverse transcriptase or template were included to assess genomic DNA and non-specific contamination, respectively.

Real-time polymerase chain reaction (PCR): The CD11b mRNA assay was performed by RT-PCR. Amplification of cDNA was performed using the QuantiTect SYBR Green PCR Kit (Qiagen, Valencia, CA) in iCycler iQ 48- well PCR plates (Bio-Rad, Hercules, CA) on a MyiQ Single Colo Real-Time PCR Detection System (Bio-Rad). The reaction mixture (12µL) was composed of 1X QuantiTect SYBR Green PCR Master Mix (containing the fluorescent dye SYBR Green I, 2.5mM MgCl2, dNTP mix, and HotStart Taq DNA polymerase), 10nM fluorescein, 500nM each of forward and reverse primers 25ng cDNA and nuclease-free H2O. The PCR amplification used primers to CD11b (forward:TCCAAGTCCGCAAGAACACCA;reverse:GACCCGGATGCGCCTGAGTAT),andGAPDH(forward:AAGATTGTCAGCAATGCATCC;reverse:ACTGTGGTCATGAGCCCTTC).

Reactions were done in triplicate (n° 3 - 5 animals/group). The reaction conditions were an initial 10 min at 95℃, followed by 40 cycles of 15s at 95℃, 15s at 60 ℃, and 25s at 72℃. Melt curve analyses were conducted to assess uniformity of product formation, primer-dimer formation, and amplification of non-specific products. Linearity and efficiency of PCR amplification were assessed using relative standard curves generated by increasing amounts of cDNA. SYBR Green 1 fluorescence (PCR product formation) was monitored in real time using the MyiQ Single Color Real-Time PCR Detection System (Bio-Rad). Threshold for detection of PCR product was set in the log–linear phase of amplification and the threshold cycle (CT, the number of cycles to reach threshold of detection) was determined for each reaction. The levels of the

target mRNAs were quantized relatively to the level of its reference gene glyceraldehyde-3-phosphate-dehydrogen-ase (GAPDH) using the comparative CT (ΔCT) method [18].

Results

A single i.pl. injection of PGE_2 (100ng/50µL) induced acute mechanical hyperalgesia 3h after its injection when compared with the saline control group, measured by Randall-Sellito (Fig. 1A, T- test, $p<0.05$). The acute hyperalgesia were completely solved 24 h after PGE_2 administration. However, after repeated PGE_2 injection for 3 days the hyperalgesia didn´t return to baseline (Fig 1B, $p<0.05$, ANOVA, Tukey test). Rats treated with PGE_2 during fourteen days presented, four days after the disruption of the PGE_2 treatment, a persistent hyperalgesia (Fig 1C, $p<0.05$, ANOVA, Tukey test). Following, spinal microglial cells participation in the PGE_2-induced acute and persistent hyperalgesia was tested. Pretreatment with intrathecal injection of Minocycline (100µg/10µL) inhibited the PGE_2-induced mechanical acute hyperalgesia measured 3h after PGE_2 injection (Fig 2A, $p<0.05$, ANOVA, Tukey test). However, pretreatment with Minocycline was not able to inhibit the persistent hyperalgesia, measured 3days after fourteen days of PGE_2 injection disruption (Fig 2B, $p<0.05$, ANOVA, Tukey test). In order to confirm the participation of spinal cord microglial cells activation on PGE_2-induced mechanical hyperalgesia, we assessed CD11b mRNA levels, a microglial activation marker. Intraplantar PGE_2 injection markedly increased the CD11b mRNA expression levels in lumbar dorsal cord collected 48h after PGE_2 injection (Fig.3A, $p<0.05$, ANOVA, Tukey test) and pretreatment with intrathecal Minocycline inhibited this increase (Fig 3B, $p<0.05$, ANOVA, Tukey test).

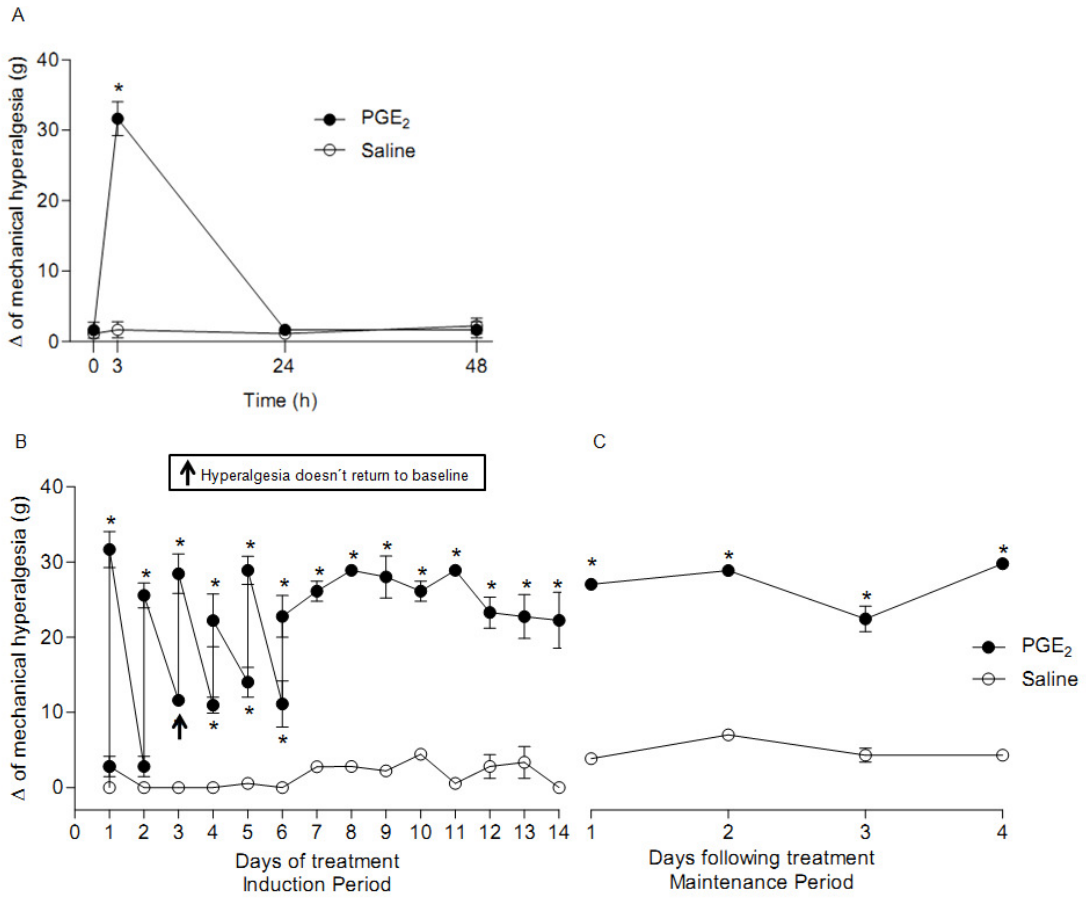

Figure 1. ***PGE$_2$-induced acute and persistent hyperalgesia.*** (A) A simple PGE_2 injection induces acute hyperalgesia 3h after treatment which is completely solved in 24 hours. (B) Intensity of hyperalgesia induced by daily i.pl. PGE_2-injections measured before (x-axis, down ticks) and 3 h after (x-axis, up ticks) PGE_2 (100ng/50µL) or saline (50µL) injection for fourteen days during induction period. Around 3th day after beginning of treatment (arrow), hyperalgesia doesn´t return to baseline. (C) Persistent hyperalgesia measured once a day during the 4 days following the discontinuation of fourteen daily of PGE_2 or saline injections. The data are expressed as means ± S.E.M.; 4-6 animals/group. The symbol (*) indicates significant difference between PGE_2 induced hyperalgesia groups and respective saline groups ($P<0.05$, unpaired two-tailed Student's t-test).

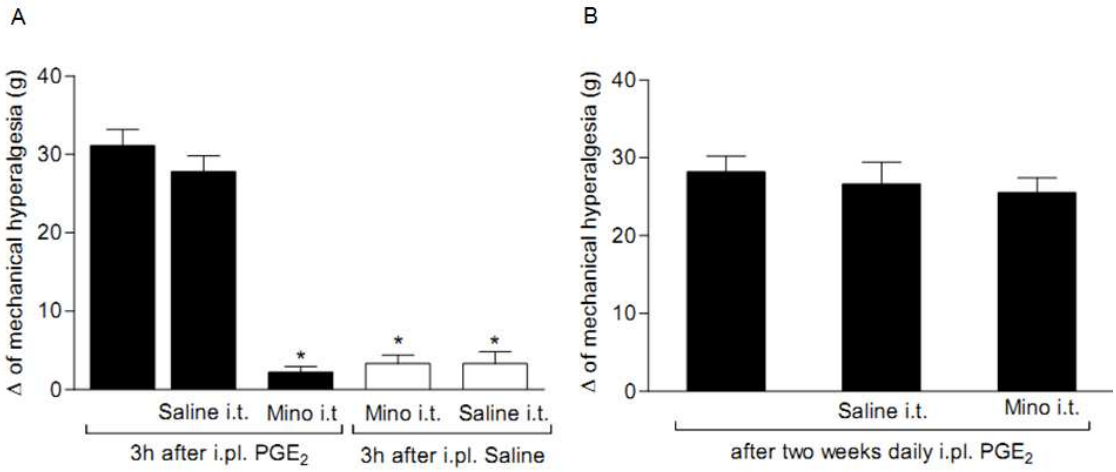

Figure 2. ***Effect of minocycline on PGE$_2$-induced acute and persistent hyperalgesia***. (A) Pretreatment with Minocycline (100µg/10µL/i.t.) inhibits PGE$_2$-induced hyperalgesia. The symbol (*) indicates a mechanical hyperalgesia significantly different when compared to PGE$_2$ (column 1) or PGE$_2$ + Saline i.t. (column 2) induced hyperalgesia ($P<0.05$, ANOVA, Tukey test). (B) Pos-treatment with Minocycline didn´t abolish the persistent hyperalgesia ($P>0.05$, ANOVA, Tukey test). The data are expressed as means ± S.E.M.

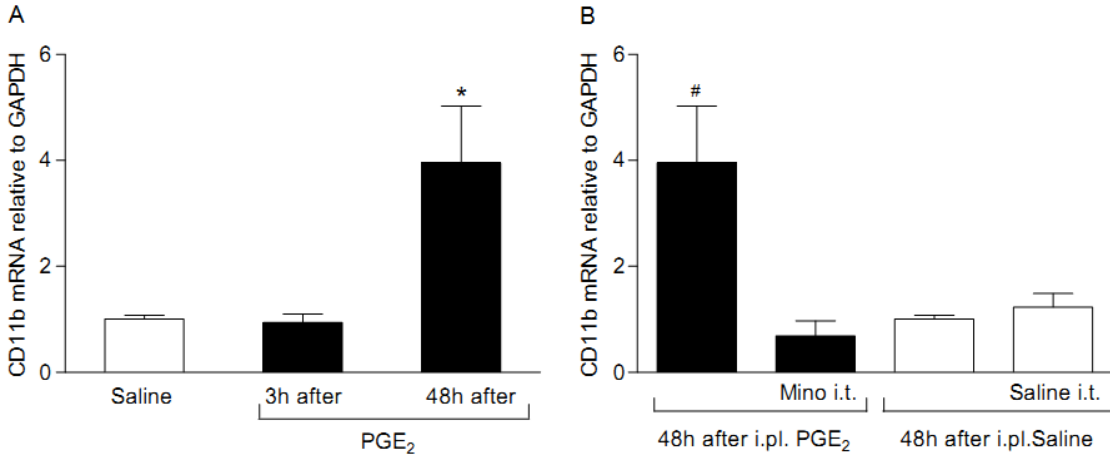

Figure 3. ***Effect of intrathecal Minocycline on PGE$_2$-induced CD11b mRNA expression in lumbar dorsal spinal cord.*** (A) Intraplantar injection of PGE_2 induced an increase of CD11b mRNA expression in spinal cord collected 48h after treatment. This increased effect didn´t occur in spinal cord collected 3h after the treatment with PGE_2. The symbol (*) indicates that hyperalgesia measures are significantly different ($P<0.05$, ANOVA, Tukey test). (B) Pretreatment with Minocycline (100µg/10µL/i.t.) during 3 days before subcutaneous PGE_2 inhibited the PGE_2-induced CD11b mRNA expression in spinal cord collected 48h after PGE_2 injection. The symbol (#) indicates significant differences in relation to PGE_2+Mino (column 2), Saline (column 3) and Saline+Saline, $p<0.05$, ANOVA, Tukey test). The data are expressed as means ± S.E.M.

Discussion

While previous studies examining the mechanisms of PGE_2-induced hyperalgesia are focused primarily on the sensorial neuronal plasticity [2, 7, 20, 37, 38], the present study suggests the involvement of central mechanisms mediated by spinal microglial cells in PGE_2-induction hyperalgesia model.

In agreement with previous studies, our results demonstrated that successive daily intraplantar injection of PGE_2 induced persistent mechanical nociceptor hypersensitivity [8, 37, 38]. It was suggested that this model could be useful in understanding the mechanism of persistent pain and, consequently, to develop new therapies in the treatment of chronic pain in humans [38].

Particularly, in the present work it was demonstrated that basal nociceptive threshold decrease around 48 hours after repeated intraplantar injection of PGE_2 during 3 days. In addition, the results demonstrated that a single intraplantar injection of PGE_2 was able to increase the mRNA expression of microglial marker, CD11b, in the lumbar spinal cord 48 hours later. These data suggest that microglial cells play an important role in the reduction of nociceptive threshold during the induction period of hyperalgesia.

Data from literature demonstrated that hyperactive microglia in spinal cord is directly correlated with development of hyperalgesia or allodynia [3, 4, 10, 17, 31, 34, 35, 24]. When pain processing is enhanced by inflammation or damage to peripheral tissues or peripheral nerves, signals can be relayed from sensory nerves to spinal glial cells to cause microglial activation. Microglial activation and its associated proinflammatory cytokines release are now strongly implicated in the creation and maintenance of enhanced pain states [3, 9, 11, 19, 23, 26, 31, 39]. Minocycline, a second-generation semisynthetic tetracycline, has been shown to exert biological effects distinct from its antimicrobial action [14]. It has emerged as a potent inhibitor of microglial activation and proliferation, without any known direct action on neurons [1, 32, 33]. Studies has been demonstrated that administration of Minocycline either systemically or intrathecally attenuated mechanical hyperalgesia and ⁄or allodynia in rat models of neuropathy mediated by the inhibition of spinal microglial activation and expression of proinflammatory cytokines [27,16]. Our results demonstrated that pretreatment with intrathecal injection of Minocycline on spinal cord was able to inhibited the induction of PGE_2-induced hyperalgesia, but pos-treatment with intrathecal injection of Minocycline didn´t revert the installed hyperalgesia (maintenance period). In addition, considering that intrathecal injection of Minocycline inhibit the expression of PGE_2-induced mRNA of CD11b, these results indicate that spinal microglia cells activation play an important role in the initial creation of enhanced nociceptive states, especially in the modulation of persistent pain states.

Corroborating our data, previous study demonstrated that intrathecal Minocycline produced a dose-dependent reduction of formalin-evoked second-

phase flinching behavior in rats, and prevented thermal hyperalgesia induced by Carrageenan injection into the paw [13]. Otherwise, it has been shown that the effect of Minocycline intrathecal administration is associated with decreased microglial activation and inhibits, but did not significantly reverse, low threshold mechanical allodynia, as measured by von Frey test in models of pain facilitation. In contrast, the same study, using a rat model of neuropathic pain induced by sciatic nerve inflammation, Minocycline delayed the induction of allodynia in both acute and persistent paradigms [16]. Several studies demonstrated that Minocycline analgesic activity was associated with inhibition of spinal microglia cells and subsequent pro-inflammatory cytokines released in the spinal cord [16, 27]. For instance, Minocycline decreased microglial activation as assessed by immunohistochemistry for CD11b or other microglial markers in cerebral ischemia models [43, 44] and Parkinson's disease [12, 42]. Moreover, Minocycline annulled the increased expression of OX-42 (a activated microglial marker) in the ventral posterolateral nucleus of the thalamus after chronic constriction injury of the sciatic nerve [15].

The prevention of a long-lasting hyperalgesic state is crucial in order to avoid the development of persistent hyperalgesia, which may be an important contributing factor to chronic pain [30]. The fact that Minocycline produces multiple effects potentially useful linked with its action on PGE_2-induced hyperalgesia, open the possibility of its new uses in neurology. Further research is now necessary to investigate direct or indirect actions of Minocycline preventing the PGE_2-induced paw hyperalgesia. Although the mechanisms underlying PGE_2-induced persistent hyperalgesia are not clear, the present observations suggest that microglial cells also participate in the signaling pathway of the persistent hyperalgesia induction.

Conclusion

In conclusion the results obtained in the present study suggest that PGE_2-induced hyperalgesia can be mediated, at least in part, by central mechanisms involving microglial cells. These results indicate that spinal microglia cells activation play an important role in the initial development of

enhanced nociceptive process, especially in the modulation of persistent pain states.

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Capítulo 2

The role of spinal microglial p38 MAPK on PGE_2 paw hyperalgesia

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Abstract

Considering that Prostaglandin E_2 (PGE_2)-induced acute hyperalgesia depends on the spinal microglial cells activation, the aim of the present work was to evaluated the intracellular mechanism of spinal microglial p38 mitogen-activated protein kinase (MAPK) in the PGE_2-induced acute and persistent paw hyperalgesia, measured by Randall & Selito test. Subcutaneous PGE_2 (100ng/50µL) injection into the hind paw evoked phosphorylation of p38 (P-p38) in the lumbar spinal cord as assessed by Western Blotting. P-p38 levels significantly increased just 3 hours after intraplantar PGE_2 injection. However, 3 days after cessation of 2 weeks of daily intraplantar treatment with PGE_2, it was not observed an increase in phosphorylated p38 MAPK. Immuncytochemisty, double-staining with markers for microglia and astrocytes shown co-localization between phosphorylated p38 MAPK-immunoreactive cells and microglia, but not with other glial cells. These results indicate that spinal microglial p38 MAPK can be a key of intracellular signal to activated spinal microglia cells during the PGE_2-induced acute paw hyperalgesia.

Keywords: p38 mitogen-activated protein kinase, microglia, hyperalgesia, prostaglandin E_2.

Introduction

Mitogen-activated protein kinases (MAPKs) play an important role in signal transduction from the cell surface to the nucleus (Ji & Suter, 2007). MAPKs are a group of enzymes in the MAPK family, including extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 (Suter, 2007). Theses enzymes are phosphorylated and activated by a variety of physical and chemical stimuli (Obata et al. 2000). Once activated, these MAPKs regulate responses, such as cells differentiation, apoptosis and inflammation-induced cytokine production (Ji & Suter, 2007).

Following that it was demonstrated that the MAPKs, p38 and ERK, are activated in spinal microglia cells under injury conditions and are important for spinal microglial signaling linked to pain states (Ji et al., 2007). Particularly, p38 MAPK is associated with spinal cord dorsal horn sensitization (Svensson et al., 2003; Xie et al., 2007). It was suggest that phosphorylated p38 MAPK in spinal cord plays a critical role in nerve injury and inflammation-induced spinal pain processing (Ji et al., 2002; Jin et al., 2003; Schafers et al., 2003; Svensson et al., 2003, 2005; Tsuda et al., 2003).

In this way, previously we demonstrated that PGE_2-induced hyperalgesia depends, at least in part, of spinal microglia cells activation (Urtado et al, 2012). Considering that studies have shown that in models of neuropathic and inflammatory pain, p38 MAPK is only phosphorylated in spinal microglia activated (Jin et al., 2003, Hains & Waxman, 2006; Hua et al., 2005; Kim et al., 2002; Svensson et al., 2003; Svensson et al., 2005; b Xu et al., 2007a; Xu et al., 2007b Tsuda et al., 2004; Wen et al., 2007), the aim of this study was to evaluate the involvement of spinal microglial p38 MAPK on PGE_2-induced paw hyperalgesia.

Material and methods

Animals: This study was carried out with adult male Wistar rats (200-300g) maintained in a temperature-controlled room (23° ± 1°C) with a 12-hour light–dark cycle. All experiments were conducted in accordance to the International

Association for the Study of Pain guidelines on using laboratory animals for investigations of experimental pain in conscious animals (Zimmermann, 1983). All animals experimental procedures and protocols were approved by the Committee on Animal Research of the University of Campinas (protocol number 1574-1). The animals suffering and number per group were kept at a minimum (4-6 animals per group).

Experimental Design: Rats were treated with daily intraplantar (i.pl.) injection of PGE_2 (100ng/50µL) or saline (50µL) during fourteen days (induction period) (Ferreira et al., 1990; Villarreal et al., 2009). The mechanical nociceptive threshold was assessed before (baseline) and 3 hours and 48 hours after the i.pl. injection of PGE_2 during the induction period and 3 days following the discontinuation of PGE_2 treatment (maintenance period). In order to avoid the local release of prostaglandins triggered by the trauma of i.pl. injection, all animals were treated with Indomethacin (2mg/Kg, intraperitonial) 30 min. before the i.pl. injection ((Ferreira et al., 1990). To evaluate the p38 MAPK spinal microglial activation on the PGE_2-induced acute and persistent paw hyperalgesia, after behavioral nociception test animals were killed and lumbar spinal cord was collected for assessed phosphorylated p38 (P-p38) and p38 expression and localization by Western Blot and Immunocytochemistry analysis. The drugs used in this study were obtained from Sigma-Aldrich, MO, USA and was dissolved in sterile saline.

Intraplantar Injection: PGE_2 or its vehicle were subcutaneously injected in the dorsum of the rat's hind paw by tenting the skin and puncturing it with a 30-gauge needle prior to injecting the test agent, as previously described (Oliveira et al., 2007). The needle was connected to a catheter of polyethylene and also to a Hamilton syringe (50µL). The animals were briefly restrained and the total volume administered in the paw was 50µL.

Mechanical paw-withdrawal nociceptive threshold test: Testing sessions took place during light phase (between 09:00 A.M. and 5:00 P.M.) in a quiet room

maintained at 23°C (Rosland et al., 1991). The Randall–Selitto nociceptive paw-withdrawal flexion reflex test (Randall & Sellito, 1957) was performed using an Ugo-Basile analgesymeter (Stoelting, Chicago, IL, USA), which applies a linearly increasing mechanical force to the dorsum of the rat's hind paw (Oliveira et al., 2007). The nociceptive threshold was definite as the force in grams, with which the rat withdrew its paw. The basal paw-withdrawal threshold was defined as the mean of three measures performed at 5-min intervals before the first i.pl. injection of PGE_2. The variation (Δ) of mechanical hyperalgesia expressed on the graphs were calculated by subtracting the mean of three consecutive measurements after the treatment evaluated (test) from the basal threshold (Δ of mechanical hyperalgesia = basal – test).

Immunocytochemistry: 3h and 48h after PGE_2 injection (induction period) and 3days after two weeks PGE_2 injection (maintenance period) or vehicle, the animals were deeply anesthetized with a mixture of Urethane Ethyl Carbamate-Sigma (100mg/Kg, i.p.) and Alfa Chloralose Sigma (50mg/Kg, i.p.) and perfused intracardially with heparinized saline (200 ml) followed by freshly prepared 4% paraformaldehyde in 0.1 M PBS, pH 7.4 (Sigma). The lumbar spinal cord was removed, post-fixed in the same fixative for 6 h, and transferred to PBS containing 20 % sucrose for 72 h at 4°C. The lumbar segments L5–6 were dissected, and transverse sections (10 µm) were cut with a freezing microtome and mounted on silane-covered glass slides. Sections from vehicle-treated and PGE_2-treated animals, mounted side by side, were incubated in polyclonal rabbit-P-p38 (1:100 dilution with 0.1%Triton X-100 and 1% BSA in PBS; Cell Signaling Technology) for 2h at room temperature. Binding sites were visualized with anti-rabbit IgG antibodies conjugated with Alexa-488 (1 : 400 dilution with 0.1% Triton X-100 and 5% 1% BSA in PBS; Invitrogen). To determine the cellular distribution of P-p38 MAPK, astrocytes and microglia were counterstained with primary antibodies raised in mouse against , glial fibrillary acid protein (GFAP) (1 : 200; Santa Cruz Biotechnology) and CD11b (OX-42) (1 : 200; BD Bioscience) respectively. Binding sites were visualized with anti-goat IgG antibody conjugated with Alexa-633 (1 : 400 with 0.1% Triton X-100, 5%

and 1% BSA in PBS; Invitrogen)and anti-mouse IgG antibody conjugated with Alexa 546 (1 : 400 with 0.1% Triton X-100, 5% and 1% BSA in PBS; Invitrogen). Non-specific staining was determined by excluding the primary antibodies. Images were captured using a Confocal laser scanning microscopy (CLSM)

Western Blot: Rats were deeply anesthetized then decapitated, and the spinal cords were ejected from the vertebral column by means of a saline-filled syringe. The lumbar part of the spinal cord was immediately homogenized in extraction buffer (50 mM Tris buffer, pH 8.0, containing 0.5% Triton X-100, 150 mM NaCl, 1 mM EDTA, phosphatase and protease inhibitors) by sonication. The tissue extracts were subjected to denaturing Nu┘ PAGE 4–12% Bis–Tris gel electrophoresis and then electrophoretically transferred to nitrocellulose membranes (Micronic Separation Inc. Westbor- ough, MA, USA). After blocking non-specific binding sites with 5% low-fat milk in phosphate-buffered saline (PBS) containing 0.1% Tween 20 for 1 h in room temperature, the membranes were probed with antibodies overnight at 4℃. After washing, the antibody–protein complexes were probed with appropriate secon- dary antibodies labeled with orseradish peroxidase for 1 h at room temperature, and detected with chemiluminescent reagents. The nitrocellulose membranes were stripped with Re-Blot western blot recycling kit (Chemicon, Temecula, CA, USA) and reblotted with different antibodies. The antibodies used detect P-p38 MAPK (1:1000), total p38 MAPK (1:1000) (Cell Signaling Technology, Beverly, MA, USA). The intensity of immunoreactive bands was quantified using ImageQuant software (Molecular Dynamics, Sunnyvale, CA, USA). P-p38 and total p38 immunopositive bands were normalized relative to ponceau (Romero-Calvo et al 2010).

Results

A single i.pl. injection of PGE_2 (100ng/50µL/paw) induced acute mechanical hyperalgesia 3h after its injection when compared with the Saline control group, measured by Randall-Sellito (Fig. 1A, T- test, p= 0.02). The acute

hyperalgesia were completely solved 48h after PGE_2 injection (hyperalgesia induction period). Otherwise, rats treated with PGE_2 during fourteen days presented, 3 days after the disruption of the PGE_2 treatment, a persistent hyperalgesia when compared with Saline control group (Fig 1B $p<0.005$, One way ANOVA and post hoc Tukey test) (hyperalgesia maintenance period). Considering that PGE_2-induced acute hyperalgesia, at least in part, by the activation of spinal microglial cells (Urtado et al., 2012), the participation of spinal microglial p38 MAPK in the PGE_2-induced acute and persistent hyperalgesia was tested. Subcutaneous PGE_2 (100ng/50µL) injection into the hind paw evoked phosphorylation of p38 (P-p38) in the lumbar spinal cord as assessed by Western Blotting. P-p38 levels significantly increased just 3 hours after PGE_2 injection (Fig 2 $p<0.005$, One way ANOVA and post hoc Tukey test). To confirm the cellular location of P-p38, spinal cord sections from PGE_2-injected rats were collected 3h after the treatment, and incubated with antibodies against P-p38, astrocytes and microglia cellular markers. Confocal images reveled the majority co localization of P-p38 immunoreactivity and CD11b microglial marker (Fig 3D, D´)

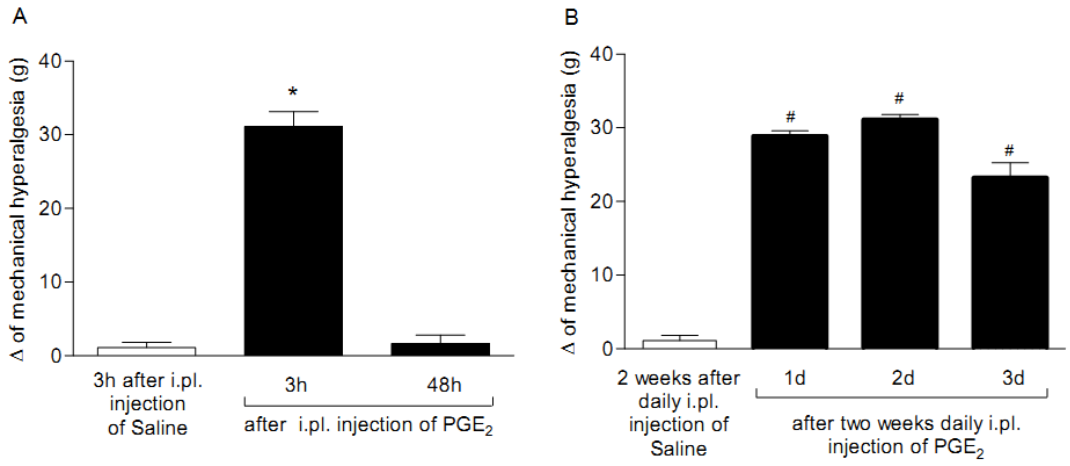

Figure 1: Acute and persistent hyperalgesia induced by an injection of PGE_2. (A) A single injection of PGE_2 induces acute paw hyperalgesia 3h after treatment which is completely solved in 48h. The symbol (*) indicates significant differences between PGE_2- induced hyperalgesia measured 3 hours after treatment compared to the other groups ($p<0.05$, One way ANOVA and post hoc Tukey test). (B) Persistent hyperalgesia induced by two weeks of daily PGE_2 injection (100 ng/ 50 µl). The data represent the behavioral mechanical response at the 1^{th}, 2^{th} and 3^{th} day after the last PGE_2 injection. The symbol (#) indicates a significant increase in the hyperalgesia in relation to Saline groups ($p<0.05$, One way ANOVA and post hoc Tukey test). The data are expressed as means ± S.E.M., 4-6 animals/group.

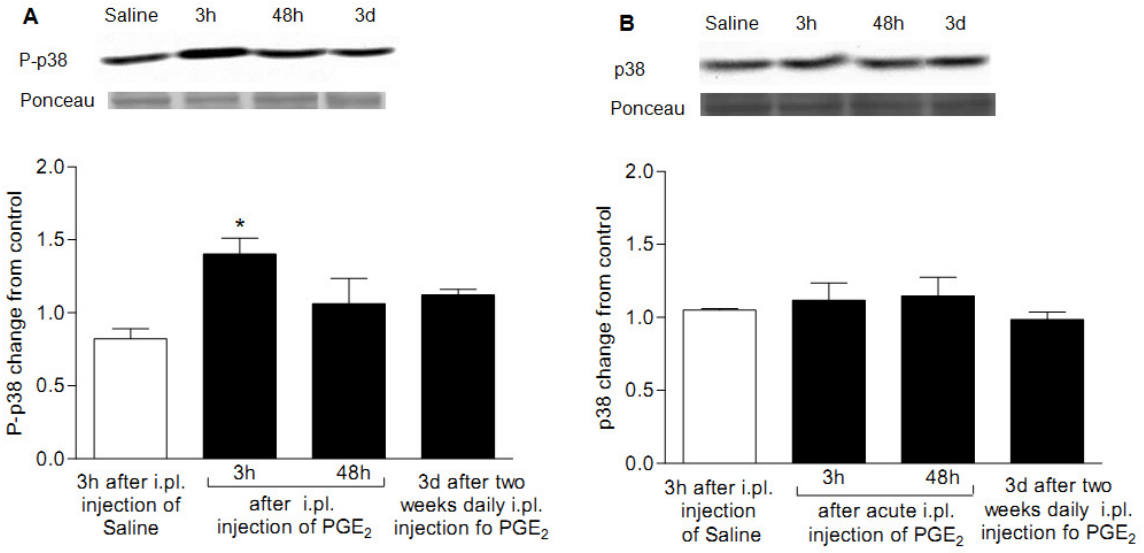

Figure 2: Subcutaneous PGE_2 injection induced p38 activation in lumbar spinal dorsal horn. (A) PGE_2 -induced in the lumbar spinal cord significantly increase the expression of P-p38 just 3h after PGE_2 injection. The symbol (*) indicates a significantly higher expression of P-p38 ($p<0.05$, One way ANOVA and post hoc Tukey test) then that other groups. (B) PGE_2-induced the expression of p38 in the lumbar spinal cord but statistical analysis demonstrated no difference between groups ($p>0.05$, One way ANOVA and post hoc Tukey test). The data are expressed as means ± S.E.M., 3 animals/group.

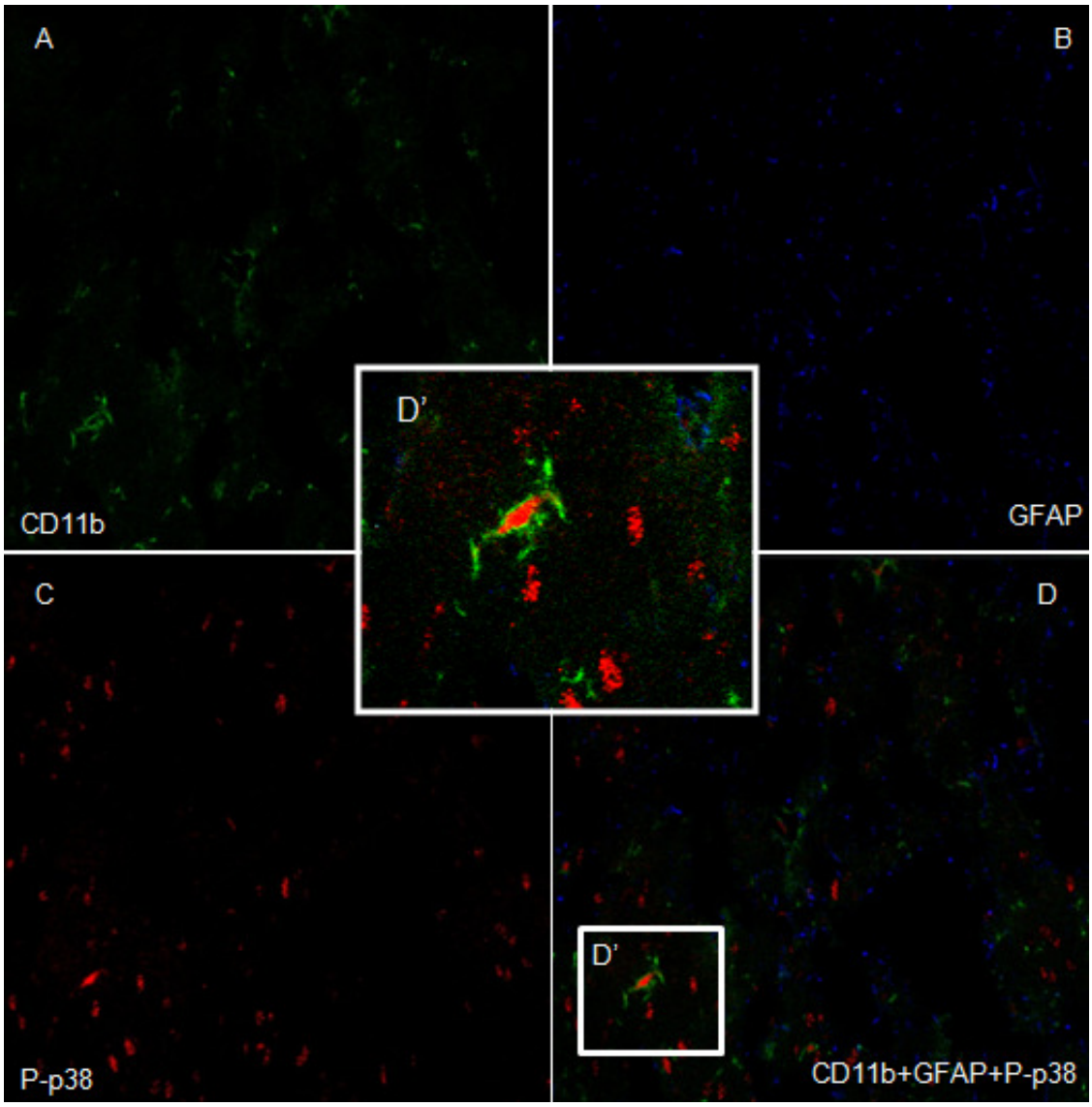

Figure 3 Co-localization of P-p38 MAPK and microglial cells. Confocal images depicting co-localization of P-p38 MAPK and microglia-like structures, but not astrocytes structures, in spinal dorsal horn 3h after intraplantar PGE_2 injection. Sections were triple-labeled with (A) CD11b, a microglia antibody recognizer (green); (B) GFAP, an astrocyte marker (blue); and (C) anti-P-p38 MAPK (red). CD11b and P-p38 MAPK labeling showed overlap (D, D´), indicating that spinal microglia p38 was phosphorylated 3h after intraplantar PGE_2 injection. No co-localization was observed between P-p38 MAPK and astrocytes.

Discussion

In the present study we suggest that activation of spinal microglial cells in PGE_2-induction paw hyperalgesia model was related with phosphorylation of spinal microglial p38 MAPK. Our results demonstrate that p38 MAPK activation occurs just 3 hours after a single intraplantar injection of PGE_2 (induction period). However, 3 days after cessation of 2 weeks of daily intraplantar treatment with PGE_2 (maintenance period), it was not observed an increase in phosphorylated p38 MAPK. These results suggest that the induction, but not the maintenance of PGE_2-induced mechanical paw hyperalgesia, was related with spinal microglial p38 MAPK phosphorylation.

Corroborated our results it was demonstrated that hyperactive microglia in spinal cord is directly correlated with development of hyperalgesia or allodynia (Colburn et al.,1999; Coyle, 1998; Guo & Schluesener, 2006; Li et al., 2010; Sweitzer et al., 1999; Tsuda et al., 2003; Tsuda et al., 2003; Morgado et al., 2011). When pain processing is enhanced by inflammation or damage to peripheral tissues or peripheral nerves, signals can be relayed from sensory nerves to spinal glial cells to cause microglial activation. Microglial activation and its associated pro-inflammatory cytokines release are now strongly implicated in the creation and maintenance of enhanced pain states (Colburn et al., 1999; Fu et al., 1999; Hashizume et al., 2000; Ma et al., 2010; Milligan et al., 2002; Popovich et al., 1997; Sweitzer et al., 1999; Watkins & Maier, 2000). In addition, it was shown that the MAPKs, p38 and ERK, are activated in spinal microglia cells under injury conditions and are important for spinal microglial signaling linked to pain states (Ji et al., 2007).

Following that several evidences indicate that p38 activation plays an important role in the development of pain states (Jin et al., 2003; Tsuda et al., 2004; Hua et al 2005; Svensson C.I. et al 2005; Ji et al., 2007; Ji & Sutter, 2007; Li Kai et al 2010). It was demonstrated that p38 MAPK is activated by stress signals, such as heat shock, ultraviolet light and ischemia as well as proinflammatory cytokines (Widman et al., 1999). Moreover, many studies from different groups have shown that in models of neuropathic and inflammatory

pain, p38 MAPK is only activated in OX-42-positive microglia in the spinal cord (Jin et al., 2003, Hains & Waxman, 2006; Hua et al., 2005; Kim et al., 2002; Svensson et al., 2003; Svensson et al., 2005; Xu et al., 2007a; Xu et al., 2007b Tsuda et al., 2004; Wen et al., 2007) which is consistent with our results suggesting that p38 is only activated in CD11b-positive microglia in the spinal cord.

Previously we demonstrated that pretreatment with intrathecal injection of Minocycline (microglia inhibitor) on spinal cord was able to inhibit the induction of PGE_2-induced hyperalgesia, but post-treatment with intrathecal injection of Minocycline did not revert the installed hyperalgesia (maintenance period) (Urtado et al., 2012). Considering the present results that phosphorylation of spinal microglia p38 MAPK occurs only 3 hours after a single intraplantar injection of PGE_2 (induction period), these results indicate that spinal microglia cells activation play an important role in the beginning of enhanced nociceptive states after peripheral stimuli. According to that different studies have shown increase phosphorylated p38 MAPK in spinal microglia cells after peripheral stimuli such as intraplantar injection of formalin (Kim et al., 2002; Li Kai et al, 2010) or carrageenan (Hua et al., 2005).

Taken together our findings strongly suggest that phosphorylation of spinal microglial p38 MAPK can be a key of intracellular signal to activated spinal microglia cells during the PGE_2-induced acute paw hyperalgesia confirming a critical role of this non-neuronal cell type in spinal pain processing.

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IV CONCLUSÃO

Os resultados do presente estudo sugerem que a hiperalgesia induzida pela injeção intraplantar de PGE_2 é mediada pelas células microgliais da medula espinhal. Especificamente para a fase de indução da dor, os resultados sugerem que a fosforilação da p38 MAPK age como um sinalizador intracelular para ativação das células microgliais. Sendo assim, pode-se concluir que a microglia espinhal tem um importante papel no processamento da dor sendo um alvo promissor na prevenção e modulação das condições dolorosas persistentes.

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ANEXO

Certificado da Comissão de Ética

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CEEA/Unicamp

C E R T I F I C A D O

Certificamos que o Protocolo nº **1574-1**, sobre **"Participação de células microgliais na instalação e manutenção da hiperalgesia persistente induzida na pata de ratos"**, sob a responsabilidade de **Profa. Dra. Maria Cecília Ferraz de Arruda Veiga / Marília Bertoldo Urtado**, está de acordo com os Princípios Éticos na Experimentação Animal adotados pelo Colégio Brasileiro de Experimentação Animal (COBEA), tendo sido aprovado pela Comissão de Ética na Experimentação Animal – CEEA/Unicamp em **05 de agosto de 2008**.

C E R T I F I C A T E

We certify that the protocol nº **1574-1**, entitled **"Participation of microgliais cells in the installation and maintenance of persistent hyperalgesia induced in the paw of rats"**, is in agreement with the Ethical Principles for Animal Research established by the Brazilian College for Animal Experimentation (COBEA). This project was approved by the institutional Committee for Ethics in Animal Research (State University of Campinas - Unicamp) on **August 5, 2008**.

Campinas, 05 de agosto de 2008.

Profa. Dra. Ana Maria A. Guaraldo
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Confirmação de Envio do Artigo para publicação (Capítulo 1)

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