Universidade Estadual de Londrina

THACYANA TEIXEIRA DE CARVALHO

DOADOR DE ÂNION SUPERÓXIDO INDUZ HIPERALGESIA MECÂNICA E CONTORÇÕES ABDOMINAIS DEPENDENTES DA ATIVAÇÃO ESPINAL DE PI_3K, MAP QUINASES E CÉLULAS DA GLIA EM CAMUNDONGOS

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Orientador: Prof. Dr. Waldiceu Aparecido Verri Junior

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BANCA EXAMINADORA

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Orientador: Prof. Dr. Waldiceu Aparecido Verri Junior
Universidade Estadual de Londrina - UEL

_____

Profª. Drª. Estefânia Gastaldello Moreira
Universidade Estadual de Londrina - UEL

_____

Profª. Drª. Gislaine Garcia Pelosi Gomes
Universidade Estadual de Londrina - UEL

Londrina, 20 de Março de 2014.

Dedico este trabalho aos meus pais, vocês sabem seu significado pra mim.

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"Há homens que lutam um dia e são bons.
Há outros que lutam um ano e são melhores.
Há os que lutam muitos anos e são muito bons.
Porém, há os que lutam toda a vida.
Esses são os imprescindíveis."

Bertolt Brecht.

CARVALHO, Thacyana Teixeira. **Doador de ânion superóxido induz hiperalgesia mecânica e contorções abdominais dependentes da ativação espinal de PI_3K, MAP quinases e células da glia em camundongos**. 2014. 65f. Dissertação (Mestrado em Patologia Experimental) – Universidade Estadual de Londrina, Londrina, 2014.

RESUMO

A inflamação é uma reação complexa do organismo a um agente nocivo que envolve componentes vasculares e celulares, atuando para a resolução do quadro e visando o retorno à homeostasia. Muitos mediadores atuam durante a inflamação para promover diferentes respostas, como as espécies reativas de oxigênio (EROs) que são produzidas pelos fagócitos com o objetivo de eliminar microrganismos fagocitados, sendo o radical ânion superóxido ($O_2^{\bullet-}$) um desses mediadores. O $O_2^{\bullet-}$ quando gerado passa por dismutação espontânea ou redução, gerando outras EROs que são mantidas sob controle pelos sistemas antioxidantes endógenos. No entanto, quando ocorre o desequilíbrio entre a geração dessas EROs e sua eliminação o organismo sofre danos celulares e teciduais provenientes dos produtos gerados a partir da interação entre os radicais livres e as células. Dessa maneira, as EROs podem ser responsáveis por desencadear um novo processo inflamatório, além de serem responsáveis pela manutenção da inflamação prévia, gerando, entre outros sinais, a dor. A dor é um sintoma debilitante para os indivíduos que sofrem de doenças inflamatórias crônicas. Recentemente, tem sido observada a participação da fosfatidilinositol-3-quinase (PI_3K), das proteínas ativadas por mitógeno (MAP) quinases e de células da glia espinais em vários modelos de dor inflamatória e neuropática. Nesse sentido, foi desenvolvido recentemente em nosso laboratório um modelo animal de inflamação induzida pela injeção de superóxido de potássio (KO_2), um dador de ânion superóxido, este modelo permite estudar os mecanismos por meio dos quais esse agente atua na inflamação. O KO_2 induz inflamação (edema da pata, recrutamento de leucócitos, comportamento semelhente à dor manifesta e hiperalgesia) e estresse oxidativo em camundongos. Além disso, há evidências de que as vias de sinalização: PI_3K e MAP quinases induzem a produção de EROs, como o $O_2^{\bullet-}$. E ainda, as células da glia produzem EROs além de serem ativadas pelo $O_2^{\bullet-}$. Assim, no presente estudo, a participação espinal da PI_3K, das MAP, ERK (quinase regulada por sinal extracelular), JNK (quinase c-Jun N-terminal) e p38, e das células da glia, microglia e astrócitos, foi investigada nos modelos do teste da pressão crescente na pata e de contorções abdominais induzidas pelo KO_2 em camundongos. A administração de KO_2 induziu significativa hiperalgesia mecânica e contorções abdominais nos camundongos, as quais foram inibidas pelo pré-tratamento por via intratecal (i.t.) com os inibidores específicos: wortmanina (PI_3K), PD98059 (ERK), SB202190 (p38), SP600125 (JNK), minociclina (microglia) e fluorocitrato (astrócitos). Além disso, o co-tratamento com os inibidores das MAP quinases e PI_3K, em doses que foram ineficazes como tratamento único, inibiu significativamente a hiperalgesia induzida pelo KO_2. Juntos, esses resultados demonstram que o ânion superóxido gerado/fornecido pela administração do KO_2 induz hiperalgesia mecânica e contorções abdominais dependentes da ativação espinal de PI_3K, MAP quinases e células da glia em camundongos, assim, esse estudo pode ser utilizado como base para a descoberta de tratamentos mais eficazes e com menor incidência de eventos adversos no combate da dor inflamatória.

Palavras-chave: KO_2. Hiperalgesia. Medula espinal. PI_3K. MAP quinases. Células da glia.

CARVALHO, Thacyana Teixeira. **Superoxide anion donor induces mechanical hyperalgesia and abdominal contortions dependent on the spinal activation of PI_3K, MAP kinases, and glial cells in mice**. 2014. 65p. Dissertation (Master's degree in Experimental Pathology) – Universidade Estadual de Londrina, Londrina, 2014.

ABSTRACT

Inflammation is a complex reaction of the organism to a harmful agent that involves vascular and cellular components working to resolve the symptoms and seeking a return to homeostasis. Many mediators act during inflammation to promote different responses, such as reactive oxygen species (ROS) that are produced by phagocytes in order to eliminate phagocytized microorganisms, the superoxide anion radical ($O_2^{\bullet-}$) is one of these mediators. The $O_2^{\bullet-}$ generated go through spontaneous dismutation or reduction, generating other ROS that are kept under control by endogenous antioxidant systems. However, when these imbalance between ROS generation and elimination occurs the organism undergoes cell and tissue damage from products generated from the interaction between free radicals and cells. Thus, ROS may be responsible for triggering a new inflammatory process, besides being responsible for the maintenance of prior inflammation, generating, among other signs, pain. Pain is debilitating symptom for individuals suffering from chronic inflammatory diseases. Recently the participation of spinal phosphatidylinositol-3-kinase (PI_3K), mitogen-activated protein (MAP) kinases and glial cells in several models of inflammatory and neuropathic pain have been observed. In this sense, we have recently developed an animal model of inflammation induced by superoxide by injecting potassium superoxide (KO_2), a donor of superoxide to study the mechanisms by which this agent acts in inflammation. The KO_2 induced inflammation (paw edema, leukocyte recruitment, overt pain-like behavior and hyperalgesia) and oxidative stress in mice. Furthermore, there is evidence that the signaling pathways: PI_3K and MAP kinases induce the production of reactive oxygen species, such as $O_2^{\bullet-}$. Also, glial cells produce ROS and are activated by $O_2^{\bullet-}$. In the present study, the spinal participation of PI_3K, MAP kinases, ERK (extracellular signal regulated kinase), JNK (c-jun N-terminal kinase) and p38, and glial cells, microglia and astrocytes, was investigated in the models of increasing paw pressure and writhing in mice induced by KO_2. The administration of KO_2 induced significant mechanical hyperalgesia and writhing in mice that were inhibited by the intrathecal (i.t.) pretreatment with specific inhibitors: wortmannin (PI_3K), PD98059 (ERK), SB202190 (p38), SP600125 (JNK) minocycline (microglia) and fluorocitrate (astrocytes). Moreover, co-treatment with inhibitors of MAP kinase and PI_3K at doses that were ineffective as single treatment significantly inhibited KO_2-induced hyperalgesia. Together these results show that the superoxide anion generade/provided by the administration of KO_2 induces mechanical hyperalgesia and writhing dependent of spinal activation of PI_3K, MAP kinases and glial cells in mice, thus, this study may be used as basis for the discovery of more efficient and with lower incidence of adverse events treatments in combating inflammatory pain.

Keywords: KO_2. Hyperalgesia. Spinal cord. PI_3K. MAP kinases. Glial cells.

LISTA DE ILUSTRAÇÕES

LISTA DE ABREVIATURAS E SIGLAS

5-HT	Serotonina
AMPc	Adenosina 3',5'-monofosfato cíclico
AP-1	Proteína de ativação 1
ASICs	Canais de íons sensíveis ao ácido
ATF	Fator ativador de transcrição
bFGF	Fator de crescimento de fibroblasto básico
BK	Bradicinina
COX	Cicloxigenase
DAMPs	Padrões moleculares associados a danos
DNA	Ácido desoxirribonucleico
ERK	Quinase regulada por sinal extracelular
ERNs	Espécies reativas de nitrogênio
EROs	Espécies reativas de oxigênio
FKN	Fractalcina
GFAP	Proteína acídica fibrilar glial
GMPc	Guanosina monofosfato cíclico
GO	Glutationa oxidase
GPX	Glutationa peroxidase
GRD	Gânglio da raiz dorsal
H_2O_2	Peróxido de hidrogênio
HSF	Fator de transcrição do choque térmico
IASP	Associação internacional para o estudo da dor
IL	Interleucina
i.p.	Intraperitoneal
i.pl.	Intraplantar
i.t.	Intratecal
JNK	Quinase c-Jun N-terminal
KO_2	Superóxido de potássio
LPS	Lipopolissacarídeo
MAP	Proteínas ativadas por mitógeno
MEF	Fatores amplificadores de miócitos
MPO	Mieloperoxidase

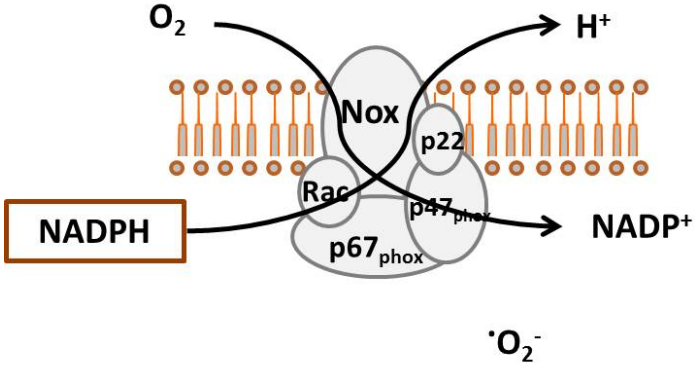
NADPH	Nicotinamida adenina dinucleotídeo fosfato
NFAT	Fator de transcrição de células T ativadas
NFκB	Fator nuclear kappa B
NO	Óxido nítrico
NOS	Óxido nítrico sintase
$O_2^{\bullet-}$	Radical ânion superóxido
$ONOO^-$	Peroxinitrito
PAF	Fator de ativação das plaquetas
PAMPs	Padrões moleculares associados a patógenos
PAR	Receptores ativados por proteases
PBQ	Fenil-p-benzoquinona
PGs	Prostaglandinas
PGE_2	Prostaglandina E 2
PI_3K	Fosfatidilinositol-3-quinase
PKC	Proteína quinase C
PRRs	Receptores de reconhecimento de padrões
Rtk	Receptor de tirosina quinase
SOD	Superóxido dismutase
SNC	Sistema nervoso central
STAT	Transdutor de sinal de transcrição
TLR	Receptores semelhantes a Toll
TNF-α	Fator de necrose tumoral alfa
TRP	Receptores de potencial transitório
TRPV1	Receptor de potencial transitório vaniloide 1
UV	Ultravioleta

SUMÁRIO

1 INTRODUÇÃO

O ânion superóxido ($O_2^{\bullet-}$) é uma espécie reativa de oxigênio (ERO), ou seja, contém um ou mais elétrons desemparelhados em sua camada de valência (HALLIWELL; GUTTERIDGE, 2006). Ele pode ser gerado por fontes como o complexo enzimático nicotinamida adenina dinucleotídeo fosfato (NADPH) oxidase (Figura 1) durante infecções, tendo como objetivo a eliminação do organismo invasor. Além disso, o ânion superóxido também tem participação em muitas doenças inflamatórias (Injúria por Reperfusão, Aterosclerose, doença de Alzheimer, doença de Parkinson) (TAUBERT et al., 2003), nas quais pode ocorrer o aumento na liberação dessa molécula e, assim, lesão em células e órgãos (JOHNSTON et al., 1975; YAN et al., 2012). Outras fontes de ânion superóxido são: a fosforilação oxidativa mitocondrial; e os sistemas enzimáticos, como a xantina oxidase, óxido nítrico sintase (NOS), e cicloxigenase (COX) (PACHER et al. 2007).

Figura 1 - Esquema da produção do ânion superóxido pelo complexo enzimático NADPH oxidase. As subunidades componentes do complexo encontram-se separadas até que um estímulo induza seu recrutamento, translocação e ativação, assim o oxigênio molecular é convertido a superóxido.



Fonte: Elaborada pela autora

A ação do $O_2^{\bullet-}$ é controlada através de complexos enzimáticos existentes no organismo que atuam de forma a estabilizá-lo, dessa maneira, outras enzimas podem atuar para eliminar os produtos dessa reação sem que haja danos ao organismo. A enzima superóxido dismutase (SOD) dismuta o ânion superóxido gerando peróxido de hidrogênio (H_2O_2) e oxigênio (O_2) através da seguinte reação (HALLIWELL; CLEMENT; LONG, 2000): $2\ O_2^- + 2\ H^+ \xrightarrow{SOD} H_2O_2 + O_2$.

O papel do $O_2^{\bullet-}$ na inflamação inclui o dano celular e aumento da permeabilidade vascular (DROY-LEFAIX et al., 1991), liberação de citocinas (MATATA; GALINANES,

2002; SALVEMINI et al., 1999), recrutamento de neutrófilos para o local da inflamação (BOUGHTON-SMITH et al., 1993; SALVEMINI et al., 1999), entre outros.

A dor é um dos sinais mais importantes da inflamação, ela indica que algo está afetando a integridade ou que alguma disfunção está ocorrendo no organismo (FERREIRA et al., 2009). Quando não resolvida, a dor se torna uma condição debilitante para a população, pois reduz a qualidade de vida dos pacientes, gerando dificuldade para desenvolver suas tarefas cotidianas, além de, algumas vezes, afetar o desenvolvimento de suas atividades no trabalho. Assim, é necessária a intervenção medicamentosa para amenizar este problema e melhorar a qualidade de vida desses indivíduos.

O superóxido tem sido apontado como mediador da dor (WANG et al., 2004). Durante processos inflamatórios há a liberação de diversos mediadores inflamatórios como a bradicinina (BK), serotonina (5-HT), histamina, prostaglandinas (PGs) e citocinas (FERREIRA et al., 2009), além de EROs e espécies reativas de nitrogênio (ERNs) (WANG et al., 2004). E ainda, a geração de superóxido e óxido nítrico (NO) pode ser mais prejudicial para o organismo visto que eles podem reagir entre si e gerar um radical mais nocivo aos tecidos, o peroxinitrito ($ONOO^-$) (HALLIWELL, 2001; HALLIWELL, 2006; WANG et al., 2004).

As vias de sinalização das proteínas ativadas por mitógeno (MAP) quinases podem ser ativadas ou inibidas pelas EROs (revisado por MCCUBREY; LAHAIR; FRANKLIN, 2006), e ainda, a fosfatidilinositol-3-quinase (PI_3K) e as MAP quinases ERK (proteína quinase regulada por sinal extracelular) e p38 são responsáveis pela geração de $O_2^{\bullet-}$, como observado em neutrófilos estimulados pelo fator de necrose tumoral alfa (TNF-α) (KILPATRICK et al., 2010). Além disso, estudos mostram o envolvimento da via da PI_3K sinalizando a ativação da NADPH oxidase por receptores semelhantes a Toll (TLR) (GAO et al., 2007). E ainda, as células da glia produzem $O_2^{\bullet-}$ via ativação da NADPH oxidase (BLOCK; ZECCA; HONG, 2007).

Vários trabalhos demonstraram o papel das vias de sinalização e células da glia espinais por meio da utilização de inibidores das MAP quinases, PI_3K e das células da glia espinais, em diferentes modelos de dor inflamatória, neuropática e dor no câncer (CARVALHO et al., 2011; CHOI et al., 2010; FITZSIMMONS et al., 2010; GAO; JI, 2009; MILLIGAN et al., 2003; OBATA et al., 2004b; PAVAO-DE-SOUZA et al., 2012; PEZET et al., 2008; SVENSSON et al., 2003; XU et al., 2007; XU et al., 2011; ZHANG et al., 2009).

Contudo, apesar da importância do ânion superóxido nessas diversas condições que apresentam dor pela ativação espinal desses mecanismos, ainda não foi demonstrado se o $O_2^{\bullet-}$ periférico induz a ativação espinal de MAP quinases e PI_3K.

2 REVISÃO DA LITERATURA

2.1 INFLAMAÇÃO

A inflamação é uma resposta muito importante na defesa do organismo contra agressões. É "uma reação complexa a vários agentes nocivos, como os microrganismos e células danificadas, geralmente necróticas, que consiste de respostas vasculares, migração e ativação de leucócitos e reações sistêmicas" (KUMAR; ABBAS; FAUSTO, 2005). Dessa forma, ela possui função protetora no controle de infecções e na promoção do reparo tecidual. No entanto, quando não ocorre a resolução da inflamação aguda e ela se cronifica, há a exacerbação do processo inflamatório que pode ser prejudicial para o organismo causando danos teciduais e doenças (ABBAS; LICHTMAN; PILLAI, 2011).

O reconhecimento de padrões moleculares associados a patógenos (PAMPs) e/ou padrões moleculares associados a danos (DAMPs) pelos seus receptores de membrana específicos, os receptores de reconhecimento de padrões (PRRs) (ABBAS; LICHTMAN; PILLAI, 2011), dá início a uma série de eventos vasculares e celulares para destruir, diluir ou isolar o agente nocivo e restaurar a homeostase, com danos mínimos ao organismo (KUMAR; ABBAS; FAUSTO, 2005).

A inflamação aguda tem início a partir do reconhecimento do estímulo pelas células residentes que são ativadas e passam a produzir citocinas pró-inflamatórias, como o TNF-α e a Interleucina (IL)-1β, e quimiocinas (p.ex., IL-8/CXCL8 – quimioatraente de neutrófilos), levando ao aumento na produção de diversos outros mediadores inflamatórios (aminas vasoativas, proteínas plasmáticas, metabólitos do ácido araquidônico, fator de ativação das plaquetas (PAF), NO, entre outros), aumento no calibre vascular com aumento no fluxo sanguíneo, influxo de proteínas plasmáticas e leucócitos, que migram de acordo com o gradiente quimiotático para o local da lesão, no qual são ativados para eliminar o agente nocivo (KUMAR; ABBAS; FAUSTO, 2005). Esta sequência de eventos promove o aparecimento dos sinais e sintomas clássicos da inflamação: Calor, Rubor, Edema e Dor.

Durante a resposta inflamatória diversos mediadores são produzidos visando combater o agente nocivo, entre eles estão as EROs, geradas durante o processo de fagocitose pelos neutrófilos e macrófagos. Esse processo ocorre devido à ativação da NADPH oxidase, que reduz o O_2, formando o $O_2^{\bullet-}$, e então, levando a geração de outras EROs pela dismutação espontânea, pela reação com ERNs ou por meio da reação com enzimas presentes nos neutrófilos, como a mieloperoxidase (MPO). Assim, os microrganismos são eliminados no fagolisossomo das células fagocíticas e as EROs são controladas pelos sistemas enzimáticos

endógenos, como a SOD, catalase, glutationa oxidase (GO) e glutationa peroxidase (GPX) (KUMAR; ABBAS; FAUSTO, 2005).

2.2 Inflamação e Estresse Oxidativo

Como mencionado anteriormente, durante o processo inflamatório ocorre a geração de diversas EROs e ERNs durante a fagocitose para a destruição dos microrganismos. Porém, quando esses mediadores são liberados em grande quantidade que exceda a capacidade do sistema antioxidante, ocorre o estresse oxidativo que provoca danos ao organismo.

O estresse oxidativo é gerado quando ocorre um desequilíbrio entre a geração e a eliminação de radicais livres. Esse processo leva a ocorrência de lesões celulares no organismo em condições patológicas. As reações mais comuns dessas espécies reativas para a promoção da lesão celular são: a peroxidação lipídica das membranas, que ocorre quando as ligações duplas dos ácidos graxos insaturados dos lipídios das membranas são atacados pelas EROs, uma interação que leva a produção de peróxidos que, por sua vez, iniciam uma reação catalítica em cadeia, podendo causar extenso dano às membranas, organelas e células; a modificação oxidativa das membranas, na qual os radicais livres promovem a oxidação da cadeia lateral dos aminoácidos, formação de ligações cruzadas entre proteínas e oxidação da estrutura principal da proteína, causando fragmentação proteica; e as lesões no ácido desoxirribonucleico (DNA), em que os radicais livres reagem com a timina no DNA nuclear e mitocondrial e causam rupturas em um dos filamentos do DNA (KUMAR; ABBAS; FAUSTO, 2005).

Além disso, as EROs geradas podem desencadear *per se* uma resposta inflamatória e, um dos sintomas dessa resposta, a dor inflamatória. Dessa maneira, é importante estudar os mecanismos pelos quais as EROs induzem a inflamação e a dor.

2.3 Dor Inflamatória

A dor é um importante mecanismo adaptativo de alerta para o indivíduo sobre um risco potencial à integridade de seu organismo (FERREIRA et al., 2009). A Associação Internacional para o Estudo da Dor (IASP) define a dor como: “uma experiência *sensorial* e *emocional* desagradável que é associada a lesões reais ou potenciais ou descrita em termos de tais lesões. A dor é subjetiva, pois cada indivíduo aprende a utilizar este termo por meio de suas experiências” (IASP, 2012).

A sensação dolorosa é proveniente da interação do estímulo doloroso (mecânico, térmico ou químico) com os receptores de membrana especializados na detecção de estímulos, de alto limiar ou alta intensidade, que estão presentes nos neurônios sensoriais nociceptivos periféricos que se encontram distribuídos nos tecidos. As fibras presentes nos neurônios são do tipo A-δ (mielinizadas - cerca de 12 a 30 m/s), que transmitem o sinal gerado pelo estímulo mais rapidamente (dor acentuada em "picada"), e C (desmielinizadas - cerca de 0,5 a 2 m/s), de transmissão lenta (dor em "queimação") (FERREIRA et al., 2009). As alterações de permeabilidade da membrana plasmática dos neurônios levam à transmissão do estímulo doloroso. Essa mudança gera um influxo iônico através de proteínas de membrana, os canais iônicos. A despolarização da membrana controla os canais, que convertem as variações iônicas em potenciais de ação, presentes na região de codificação e no axônio. Os canais podem ser ionotrópicos: como o receptor nicotínico da acetilcolina e os canais de íons sensíveis ao ácido (ASICs); ou metabotrópicos: como é o caso dos receptores acoplados à proteína G: os receptores para a bradicinina B_2, os receptores P2Y (envolvidos na potenciação da dor), os receptores para proteases (PAR), receptor de potencial transitório (TRP) - como o TRP vanilóide 1 (TRPV1) que é o receptor da capsaicina, e dos receptores de tirosina quinase (rtk) (FEIN, 2011).

Os corpos celulares dos neurônios nociceptivos aferentes primários encontram-se no gânglio da raiz dorsal (GRD) ou no gânglio trigeminal. De cada GRD sai um prolongamento axônico que se divide em dois troncos, sendo que um deles se dirige para os tecidos periféricos e o outro para a medula espinal – nervos espinais – ou para o tronco encefálico – nervos cranianos (DEVOR, 1999). Após ser ativado, o neurônio nociceptivo primário transmite a informação periférica até o corno dorsal da medula espinal ou até o núcleo trigeminal. A informação é então transmitida ao neurônio nociceptivo secundário por meio da liberação de neurotransmissores (p. ex., glutamato) pelo neurônio nociceptivo primário na fenda sináptica formada pelos dois neurônios. O neurônio nociceptivo secundário, por sua vez, conduz a informação direta ou indiretamente até os centros superiores localizados no encéfalo, nos quais são analisados e interpretados como dor (FERREIRA et al., 2009).

Assim, como a ativação neuronal periférica é carreada através da medula espinal, a liberação de mediadores nesse local pode ativar as células residentes (células da glia) e, além disso, desencadear uma resposta através da ativação das vias de sinalização e, assim, a produção de mais mediadores inflamatórios, levando à manutenção da dor.

A dor em processos fisiológicos é desencadeada por estímulos relativamente intensos e de curta duração. Por outro lado, a dor em processos de doença, exacerbada e persistente, reduz de maneira drástica a qualidade de vida dos pacientes.

Outras definições são necessárias para a compreensão do tema, como, por exemplo, o termo hiperalgesia, ou seja, o "aumento da dor a partir de um estímulo que normalmente provoca dor" (IASP, 2012).

2.4 Modelos de Avaliação da Dor Inflamatória em Camundongos

2.4.1 Teste de Contorções Abdominais

O teste de contorções abdominais é comumente utilizado para a avaliação da dor inflamatória em animais, baseando-se na administração por via intraperitoneal (i.p.) de agentes químicos que são capazes de induzir dor através da ativação ou indução da rápida produção de mediadores endógenos que sensibilizam os nociceptores (PAVAO-DE-SOUZA et al., 2012; STAURENGO-FERRARI et al., 2013; VERRI et al., 2006).

A administração do composto algogênico induz um comportamento frequente no animal que é caracterizado por contrações abdominais, movimentos do corpo como um todo (particularmente das patas traseiras), torção dos músculos dorso-abdominais e uma redução na atividade motora e incoordenação motora (LE BARS; GOZARIU; CADDEN, 2001). Isto ocorre sem que haja um estímulo térmico ou mecânico adicional.

O teste original foi descrito em 1957 por Siegmund, Cadmus e Lu, em que o estímulo utilizado era a Fenil-p-benzoquinona (PBQ), desde então, várias modificações foram feitas no teste (LE BARS; GOZARIU; CADDEN, 2001).

Este teste é muito utilizado no estágio inicial de triagem de drogas com potencial terapêutico analgésico, pois é um teste de fácil aprendizado, replicável e de rápida realização, além de não necessitar de equipamentos sofisticados (PAVAO-DE-SOUZA et al., 2012).

2.4.2 Teste da Pressão Crescente na Pata

O teste da pressão crescente na pata dos camundongos através do Analgesímetro digital (Insight®) é um método comparável ao teste dos filamentos de von Frey (VON FREY, 1896), no qual pode-se distinguir entre dois componentes da dor inflamatória: I) Sensibilização; e II) Ativação do nociceptor, tornando-o mais vantajoso do que o teste de contorções abdominais e o teste da formalina (CUNHA et al., 2004).

O Analgesímetro digital registra o peso (em gramas) decorrente da pressão exercida pelo experimentador, com o transdutor de sinal acoplado a uma ponteira de polipropileno, na pata a ser avaliada até que o camundongo realize o movimento de retirada ou sacudida (*flinch*) da pata, assim, obtêm-se a intensidade de hiperalgesia do animal devido à administração de um estímulo nocivo (p. ex.: Carragenina, PGE_2, Lipopolissacarídeo (LPS)).

2.5 Modelo de Dor e Inflamação Induzidos pelo Superóxido de Potássio (KO_2)

A partir de um doador de ânion superóxido, o superóxido de potássio (KO_2), foi padronizado em nosso laboratório, um modelo animal de dor e inflamação através de diferentes testes: pressão crescente da pata; placa quente; avaliação do edema plantar; contorções abdominais; sacudidas e tempo de lambida da pata; contagem da migração de leucócitos total e diferencial para o local do estímulo. Foi constatado que a administração do KO_2 por via intraplantar (i.pl.) induz hiperalgesia mecânica, térmica e edema de pata de maneira dose-dependente, além da migração de neutrófilos. Além disso, a administração do KO_2 por via intraperitoneal (i.p.) induz um aumento na contagem de leucócitos totais que migraram para a cavidade peritoneal, devido ao aumento de mononucleares e neutrófilos. Foi também observado que o efeito do KO_2 foi inibido pela utilização de um conhecido antioxidante (MAIOLI, N. A. et al., dados não publicados).

Esse modelo torna-se importante para a realização de estudos acerca dos mecanismos envolvidos no desenvolvimento da dor inflamatória induzida pelos radicais livres gerados durante a inflamação, para, dessa maneira, fornecer base para novos tratamentos que atuem diretamente no alvo e que possuam menor incidência de reações adversas ao medicamento.

2.6 Vias de Sinalização Intracelular

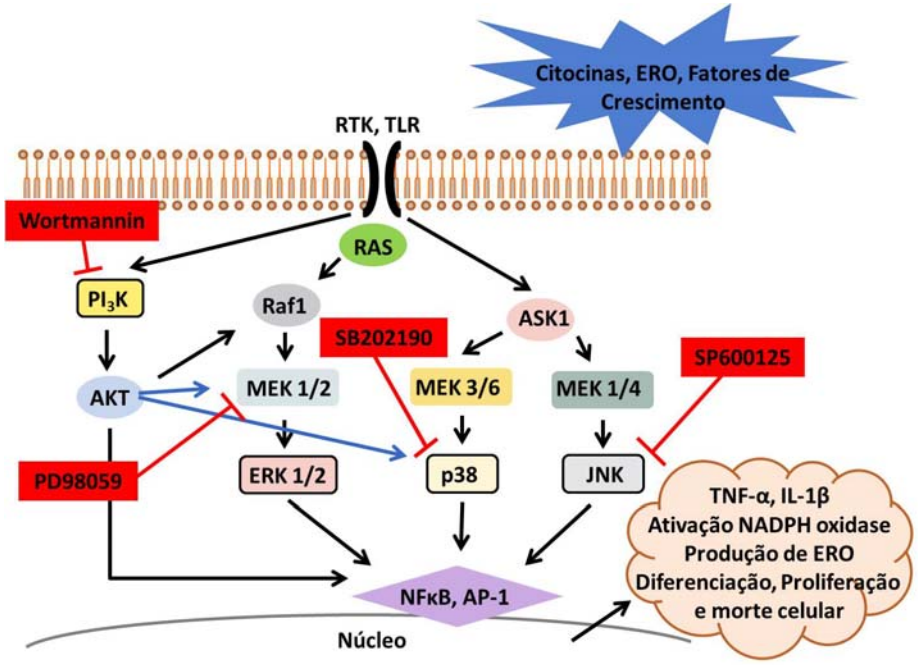
2.6.1 Fosfatidilinositol-3-Quinase

A via fosfatidilinositol-3-quinase (PI_3K) fosforila a posição D3 do anel inositol de fosfoinositídeos para a geração de moléculas de sinalização intracelular (WHITMAN et al., 1988), essas moléculas ativam efetores (p. ex.: Akt e MAP quinase ERK) (PEZET et al., 2008). Uma vez ativada, a via da PI_3K desencadeia uma resposta que leva à ativação de fatores de transcrição, como o transdutor de sinal de transcrição 3 (STAT3) (HART et al., 2011), e à respostas celulares a fatores de crescimento, como inibição da apoptose e outras (CARPENTER; CANTLEY 1996).

A wortmanina é um inibidor (figura 2) da via da PI_3K que é obtido a partir do fungo *Penicillium funiculosum*. Ele interage com a subunidade catalítica da PI_3K (p110α) (MORAND-CONTANT; ANAND-SRIVASTAVA; COUTURE, 2010; WYMANN et al., 1996). Esse inibidor é amplamente utilizado, pois é permeável a célula e tem efeitos mínimos sobre outras moléculas na concentração em que inibe totalmente a PI_3K. Não interfere com as atividades da proteína quinase C (PKC); proteínas quinase ativadas por mitógeno (MAP quinases); proteínas quinase dependentes da calmodulina, adenosina 3',5'-monofosfato cíclico (cAMP ou AMP cíclico) e guanosina monofosfato cíclico (cGMP ou GMP cíclico) (WYMANN et al., 1996).

Alguns trabalhos demonstraram que a inibição espinal da PI_3K inibiu as duas fases do teste da formalina (PEZET et al., 2008), a hiperalgesia induzida pelo fator estimulador de colônias de granulócitos (CARVALHO et al., 2011) e as contorções abdominais induzidas pelo ácido acético ou PBQ (PAVAO-DE-SOUZA et al., 2012), indicando o papel dessa via na modulação da dor e inflamação.

Figura 2 - Esquema das vias de sinalização PI3K, ERK, p38 e JNK, seus ativadores, efetores e inibidores. Um estímulo (citocinas, fatores de crescimento, EROS) atua sobre os receptores de tirosina quinase ou TLRs presentes na membrana celular de vários tipos celulares e desencadeia uma série de fosforilações das vias de sinalização que culminam na ativação dos fatores de transcrição (p. ex. NFκB, AP-1) que levam a produção de citocinas pró-inflamatórias, ativação da NADPH oxidase, além da diferenciação, proliferação e morte celular. PI_3K - fosfatidilinositol-3-quinase; ERK - Quinase regulada por sinal extracelular; RTK – Receptor de tirosina quinase; TLR – Receptor semelhante a *Toll*; EROs – Espécies reativas de oxigênio; JNK – Quinase c-Jun N-terminal; NFκB – fator nuclear κ B ; AP-1 – Proteína de ativação 1



Fonte: Elaborada pela autora.

2.6.2 Proteínas Quinase Ativadas por Mitógeno

As proteínas quinase ativadas por mitógeno (MAP quinases) são uma família de proteínas serina/treonina quinases evolutivamente conservadas que transduzem estímulos extracelulares como fatores de crescimento, hormônios, entre outros, que atuam através de diferentes receptores, como os receptores de tirosina quinase, em respostas intracelulares, como expressão gênica e síntese proteica (OBATA et al., 2004a). Assim essas quinases controlam muitos eventos celulares como a diferenciação, proliferação e morte celular (revisado em CHEN et al., 2001). Todas as MAP quinases são ativadas sequencialmente por fosforilação de diferentes MAP quinases.

A inativação dessas vias é controlada através de fosfatases que removem o grupo fosfato dos resíduos de serina/treonina ou tirosina, ou ambos.

A família das MAP quinases consiste de três membros principais: ERK, p38 e a quinase c-Jun N-terminal/proteína quinase ativada pelo estresse (JNK/SAPK).

Tem sido demonstrado que a administração intratecal (i.t.) de inibidores (Figura 2) das vias das MAP quinases ERK, p38 e JNK tem inibido o desenvolvimento de alodinia e hiperalgesia em diferentes modelos de dor (CARVALHO et al., 2011; FITZSIMMONS et al., 2010; GAO et al., 2009; KIM et al., 2002; OBATA et al., 2004a,b; PAVAO-DE-SOUZA et al., 2012; SVENSSON et al., 2003; XU et al., 2007; ZHUANG et al., 2005), sendo um de seus mecanismos a inibição da produção de citocinas (revisado em CHEN et al., 2001; OBATA et al., 2004b).

2.6.2.1 Quinase regulada por sinal extracelular

A ERK foi o primeiro membro da família identificado (JI et al., 2009) e é expressa em graus diferentes em todos os tecidos. Em fibroblastos, ela é ativada por fatores de crescimento, soro, e em uma menor extensão por ligantes de receptores heterotriméricos acoplados à proteína G, citocinas, estresse osmótico e outros (revisado em CHEN et al., 2001). Ela é ativada através de uma cascata que se inicia pela ativação de Ras que fosforila a MEK e, por sua vez, fosforila e ativa a ERK, a qual sinaliza a ativação de fatores de transcrição como a proteína de ativação (AP-1) e STAT3 (revisado em CHEN et al., 2001; KARIN, 1995; OBATA et al., 2004a). Possui papel crítico na regulação da mitose, proliferação, diferenciação e sobrevivência das células mamíferas durante seu

desenvolvimento, além de desempenhar um papel importante na plasticidade neuronal (IMPEY; OBRIETAN; revisado por JI et al., 2009; STORM, 1999; WIDMANN et al., 1999).

O PD98059 é um inibidor altamente seletivo da ativação da MEK 1 (Figura 2). Ele se liga às formas inativas da MEK 1 e previne a ativação pelos ativadores que a antecedem, como a c-Raf. Ele conhecido por inibir também a ERK, que é o alvo subsequente da cascata de fosforilação da MEK. Não inibe a ativação de outras proteínas quinase altamente relacionadas com especificidade dupla (PD98059, Datasheet).

2.6.2.2 Quinase p38

As MAP quinases p38 são uma subfamília que exibe 4 isoformas p38α, p38β, p38γ e p38δ, sendo assim, as isoformas apresentam diferentes padrões de expressão nos tecidos, sendo que uma isoforma pode ser encontrada no músculo esquelético, enquanto outra está presente nos pulmões, rins, órgãos endócrinos e intestino delgado. p38 fosforilada aumenta a atividade dos fatores amplificadores de miócitos (MEF-2A e MEF-2C), fator ativador de transcrição (ATF-1/2) (revisado em CHEN et al., 2001) e NFκB (fator nuclear κ B) (CALLEROS et al., 2006; KEFALOYIANNI; GAITANAKI; BEIS, 2006; OLSON et al., 2007). p38 é ativada por citocinas pró-inflamatórias e estresse celular e desempenha um papel essencial na regulação de respostas inflamatórias, neurodegeneração e morte celular (GAO; JI, 2008; JI; WOOLF, 2001; KUMAR et al., 2003; WIDMANN et al., 1999).

O inibidor da MAP quinase p38 SB202190 é altamente seletivo, potente e permeável à célula (Figura 2). Ele se liga à forma ativa, na qual compete com o ATP pelo mesmo sítio de ligação, e inativa da proteína. O SB202190 inibe seletivamente às isoformas α e β da p38, sem ter efeito sobre JNK, p42/44 MAP quinase (ERK) ou outras proteínas quinase (SB202190, Product Information).

2.6.2.3 Quinase c-jun-N-terminal

As quinases c-Jun N-terminal/proteína quinase é ativada pelo estresse (JNK), também conhecidas como SAPKγ, SAPKα e SAPKβ, são identificadas como proteínas do estresse, pois sua atividade aumenta em resposta à citocinas pró-inflamatórias, irradiação ultravioleta (UV), privação de fatores de crescimento e agentes que interferem na síntese de DNA e proteínas, a JNK desempenha um papel essencial na regulação de respostas inflamatórias, neurodegeneração e morte celular (GAO; JI, 2008; JI; WOOLF, 2001; KUMAR et al., 2003;

WIDMANN et al., 1999). Os fatores de transcrição ativados por essa via são, entre outros, o fator de transcrição de células T ativadas (NFAT), fator de transcrição do choque térmico (HSF-1) e STAT3 (revisado em CHEN et al., 2001).

SP600125, também chamado de Antrapirazolona, é um inibidor potente, permeável à célula, seletivo e reversível que inibe de uma maneira dose-dependente a fosforilação da JNK (Figura 2), além de inibir a expressão de genes pró-inflamatórios COX-2, IL-2, IFN-γ e TNF-α (BENNETT et al., 2001).

2.7 Células da Glia

As células da glia fazem parte do Sistema Nervoso Central (SNC), sendo essenciais aos neurônios. A neuroglia pode ser classificada em dois grupos: microglia – que tem a função de defesa do SNC, sendo recrutada então após infecções, lesões ou doenças neurodegenerativas; e macroglia – que compreende 3 tipos celulares distintos, os oligodendrócitos, os ependimócitos e os astrócitos, sendo os últimos a principal fonte de fatores de crescimento para os neurônios e presentes em diversas regiões do SNC (GOMES; TORTELLI; DINIZ, 2013).

Os astrócitos são as células da glia mais abundantes no SNC. Possuem prolongamentos com filamentos intermediários (fibrilas gliais), cujo componente principal é a proteína acídica fibrilar glial (GFAP), entre outras. Os astrócitos também auxiliam na defesa através da produção de mediadores inflamatórios como as citocinas (GOMES; TORTELLI; DINIZ; 2013).

O fluorocitrato é um inibidor metabólico reversível das células da glia que atua por meio da inibição seletiva da enzima aconitase do ciclo de Krebs glial, não atuando sobre os neurônios (HASSEL et al., 1992; JIANG et al., 2009; MILLIGAN et al., 2003; PAULSEN et al., 1987). Este inibidor tem sido utilizado como inibidor de astrócitos na medula espinal (IKEDA; KIRITOSHI; MURASE, 2012).

A microglia produz citocinas pró-inflamatórias, como a IL-1β, a IL-6 e o TNF-α (HANISCH, 2002; KOISTINAHO; KOISTINAHO, 2002; KREUTZBERG, 1996).

A minociclina é um derivado semi-sintético da tetraciclina associado com efeitos anti-inflamatórios não relacionados à sua ação microbicida, e ainda, efeitos neuroprotetores associados à inibição da microglia e não de astrócitos (TIKKA et al., 2003; YRJÄNHEIKKI et al., 1998, 1999).

Microglia e astrócitos são dois tipos de células da glia que podem ser ativadas pelos

neurotransmissores da dor, como a substância P, glutamato, entre outros (MILLIGAN et al., 2003). E ainda, há crescente evidência de que a células da glia espinais tenham um papel importante na patogênese da dor. Estudos mostram a participação da microglia e de astrócitos na dor neuropática (GAO et al., 2009; MILLIGAN et al., 2003), resposta nociceptiva induzido pelo veneno do escorpião *Buthus martensi* Karch (JIANG et al., 2009), no comportamento semelhante à dor manifesta induzido pela formalina, ácido acético e PBQ (PAVAO-DE-SOUZA et al., 2012).

3 OBJETIVOS

3.1 OBJETIVO GERAL

Avaliar se o superóxido de potássio (KO_2 – um doador de ânion superóxido) induz dor inflamatória dependente da ativação espinal de PI_3K, MAP quinases e células da glia.

3.2 OBJETIVOS ESPECÍFICOS

Avaliar a:

- Participação das MAP quinases e da PI_3K espinais na hiperalgesia mecânica induzida pela administração intraplantar (i.pl.) de superóxido de potássio (KO_2);
- Participação sinérgica da ativação das vias por meio do co-tratamento com os inibidores das MAP quinases e PI_3K, verificando a inibição da hiperalgesia mecânica induzida pela administração i.pl. de KO_2;
- Participação das células da glia espinais, microglia e astrócitos, na hiperalgesia mecânica induzida pela administração i.pl. de KO_2;
- Participação das MAP quinases e da PI_3K espinais na dor manifesta induzido pela administração intraperitoneal (i.p.) de KO_2;
- Participação das células da glia espinais na dor manifesta induzido pela administração i.p. de KO_2.

4 ARTIGO

Este é um trabalho realizado no Laboratório de Dor e Inflamação, formado pelo artigo científico: KO_2 induces mechanical hyperalgesia and overt pain-like behavior via spinal activation of PI_3K, MAP kinases, and glial cells in mice, de autoria de: Thacyana T. Carvalho, Cássia Calixto-Campos, Sandra S. Mizokami, Felipe A. Pinho-Ribeiro, Rubia Casagrande, Waldiceu A. Verri Jr.

As formatações do artigo seguem as normas do periódico *Free Radical Biology & Medicine* (Anexo). Gostariamos de ressaltar que estamos desenvolvendo experimentos de biologia molecular para adequar o trabalho ao periódico escolhido.

KO_2 induces mechanical hyperalgesia and overt pain-like behavior via spinal activation of PI_3K, MAP kinases, and glial cells in mice

Thacyana T. Carvalho[a], Cássia Calixto-Campos[a], Sandra S. Mizokami[a], Felipe A. Pinho-Ribeiro[a], Rubia Casagrande[b], Waldiceu A. Verri Jr[a,*].

Highlights

- KO_2 induced mechanical hyperalgesia via spinal PI_3K activation.
- KO_2 induced mechanical hyperalgesia via spinal MAP kinases activation.
- Abdominal contortions induced by KO_2 were reversed by PI_3K and MAP kinases inhibitors.
- Co-treatment with PI_3K and MAP kinases inhibitors reduced pain behavior in mice.
- KO_2 induced mechanical hyperalgesia and writhing via spinal activation of microglia and astrocytes.

KO_2 induces mechanical hyperalgesia and overt pain-like behavior via spinal activation of PI_3K, MAP kinases, and glial cells in mice

Thacyana T. Carvalho[a], Cássia Calixto-Campos[a], Sandra S. Mizokami[a], Felipe A. Pinho-Ribeiro[a], Rubia Casagrande[b], Waldiceu A. Verri Jr[a,*].

[a]Departamento de Patologia, Centro de Ciências Biológicas, Universidade Estadual de Londrina, Rod. Celso Garcia Cid KM480 PR445, CEP 86057-970, Cx Postal 10.011, Londrina, Parana, Brazil

[b]Departamento de Ciências Farmacêuticas, Centro de Ciências da Saúde, Universidade Estadual de Londrina, Avenida Robert Koch, 60, CEP 86039-440, Londrina, Parana, Brazil

*Author to whom correspondence should be addressed. Tel: + 55 43 3371 4979. Fax: + 55 43 3371 4387. E-mail: waverri@uel.br or waldiceujr@yahoo.com.br

Abstract

Superoxide anion ($O_2^{\bullet-}$) is produced by cells during normal and inflammatory conditions. Nevertheless, the levels of $O_2^{\bullet-}$ are much greater during inflammation and play essential roles in its development. We have recently developed a model of $O_2^{\bullet-}$-induced inflammation by injection of potassium superoxide (KO_2), a superoxide donor, that cause mechanical hyperalgesia, overt pain-like behavior response in mice. There is evidence that the signaling pathways phosphatidylinositol-3-kinase (PI_3K) and mitogen-activated protein (MAP) kinases induce the production of reactive oxygen species, such as $O_2^{\bullet-}$. Also, glial cells produce ROS (reactive oxygen species) and are activated by $O_2^{\bullet-}$. Many studies have reported the spinal participation of PI_3K, MAP kinases and glial cells in neuropathic and inflammatory pain. So, in the present study, the participation of spinal PI_3K, MAP kinases, ERK (extracellular signal-regulated kinase), JNK (c-Jun N-terminal kinase) and p38, and glial cells in KO_2-induced mechanical hyperalgesia and abdominal contortions was investigated. KO_2 administration induced significantly mechanical hyperalgesia and abdominal contortions that were inhibited by intrathecal pre-treatment with wortmannin (PI_3K), PD98059 (ERK), SP600125 (JNK), SB202190 (p38), and minocycline (microglia), and fluorocitrate (astrocyte) inhibitors.

Abbreviations

KO_2, potassium superoxide; PI_3K, phosphatidylinositol-3-kinase; MAP, mitogen-activated protein; ROS, reactive oxygen species; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase

Furthermore, the co-treatment with MAP kinases and PI_3K inhibitors, at doses that were ineffective as single treatment, significantly inhibited KO_2-induced hyperalgesia. In conclusion, superoxide anion-induced mechanical hyperalgesia and abdominal contortions depend on the spinal activation of PI_3K, MAP kinases and glial cells.

Keywords

KO_2, Superoxide anion, pain, hyperalgesia, spinal cord, PI_3K, ERK, p38, JNK, microglia, astrocytes.

Introduction

Spinal activation of phosphatidylinositol-3-Kinase (PI_3K), MAP kinases family members such as extracellular signal-regulated protein kinase (ERK), p38, and c-Jun N-terminal kinase (JNK), and microglia and astrocytes are common nociceptive mechanisms in various animal models of pain including carrageenan-induced peripheral hyperalgesia [1-3], sciatic inflammatory neuropathy (SIN) model [4], nerve lesion-induced neuropathic pain [5-7], formalin-, acetic acid-, and PBQ-induced overt-pain [8-10], CFA-induced mechanical allodynia [11], and cytokine-induced mechanical hyperalgesia [12-14]. Those kinases are activated in glial cells and neurons in the spinal cord [7,15-17] with some variations depending on the disease context, but it is clear that spinal PI_3K and MAP kinases are targets to control acute and chronic pain.

There is evidence that PI_3K and MAP kinases are involved in the production of reactive oxygen species (ROS) such as superoxide radical. For instance, TNF-α induces the production of NADPH (nicotinamide adenine dinucleotide phosphate) oxidase-derived superoxide via three pathways: a) ERK activates PKCδ that phosphorylates and recruits p47phox subunit of NADPH oxidase; b) p38 activates p47phox independently of PKCδ; and c) PI_3K activates PKCδ that phosphorylates and recruits p47phox [18]. On the opposite pathway, there is also evidence that PI_3K and MAP kinases are activated by ROS. For instance, ROS induce ERK, JNK, and p38 activation through: directly action on growth factor receptors (e. g. EGF receptor; PDGF receptor); activation of Src kinases that have a role in RAS activation; increase intracellular calcium; upstream and downstream of the TNF receptor [19]. Furthermore, ROS activate PI_3K pathway in vascular smooth muscle cells [20] and fibroblasts and epithelial cells [21]. Glial cells also produce ROS as well as are activated by superoxide [22]. Glial cells activation is crucial for the development and maintenance of acute and chronic inflammation [4,23].

The superoxide anion radical ($O_2^{\bullet-}$) is produced by the phagocyte NADPH oxidase complex [24,25] and maintained under control by the superoxide dismutase (SOD) enzymatic system in the mitochondria (Mn based), in the cytosol (Cu and Zn), or on extracellular surfaces (Cu and Zn) [24,26,27], that catalyzes the dismutation of one $O_2^{\bullet-}$ through reduction to hydrogen peroxide (H_2O_2), and other $O_2^{\bullet-}$ oxidized to molecular oxygen (O_2) [28]. Other important sources of superoxide include: mitochondrial oxidative phosphorylation and enzymatic systems such as of xanthine oxidase, NOS, and COX enzymes [29]. Under bacterial infections superoxide and other reactive oxygen species (ROS) are produced during phagocytosis in the respiratory burst to kill bacteria and restore homeostasis [24,30]. However, under acute or chronic inflammation the production of superoxide is increased at levels that lead to the quickly consumption of the endogenous SOD [31] and can cause cells and organs damage, and importantly, superoxide has a role as an endogenous signaling molecule [25,31,32]. Interestingly, superoxide has an important role in pain [31], which was mainly addressed using pharmacological tools to modify its levels. Currently it is accepted that superoxide mediates pain by direct peripheral sensitization after superoxide formation; modulates events such as neutrophil influx that in the inflammatory foci release nociceptive molecules; the release of various proinflammatory cytokines such as TNF-α, IL-1β, and IL-6; the formation of peroxynitrite ($ONOO^-$); and poly-ADP-ribose-polymerase (PARP) activation [26,31,33].

We have recently developed a model of superoxide-induced pain by injection of potassium superoxide (Maioli NA et al., under preparation). In this model, the known superoxide donor potassium superoxide induces mechanical hyperalgesia and abdominal contortions in a dose-dependent manner in morphine- and antioxidant-sensitive manner. In the present study, the role of PI_3K, MAP kinases and glial cells in potassium superoxide-induced mechanical hyperalgesia and abdominal contortions in mice were evaluated.

Materials and methods

Animals

The experiments were performed on male Swiss mice (20–25 g, Universidade Estadual de Londrina, Londrina, PR, Brazil) housed in standard clear plastic cages (six per cage) with free access to food and water. All behavioral testing was performed between 9:00 am and 5:00 pm in a temperature-controlled room. Animals' care and handling procedures were in accordance with the International Association for Study of Pain (IASP) guidelines and with the approval of the Ethics Committee of the Universidade Estadual de Londrina (OF. CIRC. CEUA-UEL No. 97/12).

KO_2 Model of inflammatory pain

The potassium superoxide (KO_2 – a superoxide anion donor), was recently introduced in our laboratory as a novel model of inflammatory pain. The KO_2 can be injected intraplantar (i.pl.) to evaluate mechanical hyperalgesia, or intraperitoneally (i.p.) inducing pain-like behavior in mice (Maioli NA et al., under preparation).

Electronic pressure–meter test for mice

Mechanical hyperalgesia was tested in mice as previously reported [34]. Briefly, in a quiet room, mice were placed in acrylic cages (12×10×17 cm) with wire grid floors, 15–30 min before the start of testing. The test consisted of evoking a hind paw flexion reflex with a hand-held force transducer (electronic von Frey anesthesiometer; Insight, Ribeirao Preto, SP, Brazil) adapted with a 0.5 mm^2 contact area polypropylene tip. The investigator was trained to apply the tip perpendicularly to the central area of the hind paw with a gradual increase in pressure. The end point was characterized by the removal of the paw followed by clear flinching movements. After the paw withdrawal, the intensity of the pressure was recorded automatically. The value for the response was an average of three measurements. The animals were tested before and after treatment. The results are expressed by delta (Δ) withdrawal threshold (in g) calculated by subtracting the zero-time mean measurements from the mean measurements (indicated time points) after stimulus. Nociceptive response was evaluated in the ipsilateral and contra-lateral paw. There was no difference of basal mechanical withdrawal thresholds between groups in the same experiment.

Writhing response tests

The KO_2 or vehicle was injected into the peritoneal cavities of mice. Each mouse was placed in a large glass cylinder and the intensity of nociceptive behavior was quantified by counting the total number of writhes (contraction of the abdominal muscle) occurring between 0 and 20 min after stimulus injection. The intensity of the writhing response was expressed as the cumulative number of writhing's in 20 min (Maioli NA et al., under preparation).

Intrathecal (i.t.) drug administration

The i.t. injections were performed under ether anesthesia. The dorsal fur of each mouse was shaved, the spinal column was arched, and a 29-gauge needle was directly inserted into the subarachnoid space, between the L4 and L5 vertebrae [35]. Correct i.t. positioning of the needle tip was confirmed by manifestation of a characteristic tail flick response. A 5 µl volume containing the test agent was slowly injected.

Drugs

Drugs used were: KO_2 (30 µg/i.pl or 1000 µg/i.p, Alfa Aesar, 96.5%, Ward Hill, MA, USA), wortmannin (0.3–3 µg/ intrathecal [i.t.]) [6,10,13,36], PD98059 (1–10 µg/i.t.) [10,13,16,36-38], SB202190 (1–10 µg/i.t.) [10,13,39], SP600125 (1–10 µg/i.t.) [10,13,40], minocycline (15-150 µg/i.t.) [10,41,42], and fluorocitrate (0.05-0.45 µg/i.t) [4,10] obtained from Sigma-Aldrich (St Louis, MO, USA). KO_2 was dissolved in saline, minocycline and fluorocitrate were dissolved in 2% DMSO in saline, and all other compounds were dissolved in 20% DMSO in saline.

General procedures

Mice were treated intrathecally (i.t.) with wortmannin (PI_3K), PD98059 (ERK 1/2), SB202190 (p38), SP600125 (JNK), minocycline (microglia) or fluorocitrate (astrocytes) inhibitors, or vehicle 30 min before intraplantar (i.pl.) KO_2 stimulus and the mechanical hyperalgesia was evaluated in ipsilateral and contra-lateral paws 30 min-7h, or before intraperitoneal (i.p.) KO_2 stimulus and the abdominal contortions was evaluated between 0 to 20 minutes.

Statistical analysis

Results are presented as means± s.e.m. of measurements made on 6 animals in each group. Two-way analysis of variance (ANOVA) was used to compare the groups and doses at all times (curves) when the hyperalgesic responses were measured at different times after the

stimulus injection. The analyzed factors were treatments, time and time versus treatment interaction. When there was a significant time versus treatment interaction, one-way ANOVA followed by Bonferroni's test was performed for each time. On the other hand, when the hyperalgesic responses were measured once after the stimulus injection, the differences between responses were evaluated by one-way ANOVA followed by Bonferroni's test. Statistical differences were considered to be significant at $P<0.05$.

Results

Intraplantar (i.pl.) administration of potassium superoxide (KO_2 - a superoxide anion donor) induces mechanical hyperalgesia via spinal PI_3K

The intrathecal (i.t.) injection of 0.3 μg of wortmannin was not capable of inhibit the KO_2-induced hyperalgesia (Fig. 1A). On the other hand, the dose of 1 μg of wortmannin inhibited the mechanical hyperalgesia 30 min-7h after stimulation (Fig. 1A). The dose of 3 μg of wortmannin inhibited KO_2-induced hyperalgesia in all times, also the higher dose was significantly compared with vehicle i.t. + KO_2 i.pl., wortmannin 0.3 μg i.t. + KO_2 i.pl., and wortmannin 1 μg i.t. + KO_2 i.pl. groups 1-5 h after stimulus injection. The wortmannin i.t. treatment did not alter the hyperalgesia in the contra-lateral paw (Fig. 1B).

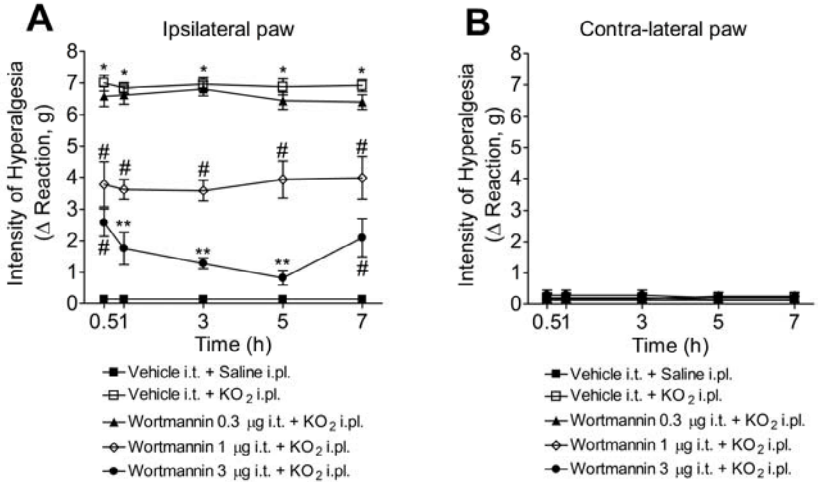


Figure 1. Intraplantar (i.pl.) administration of potassium superoxide (KO_2 - a superoxide anion donor) induces mechanical hyperalgesia via spinal PI_3K. The intrathecal (i.t.) treatment with PI_3K inhibitor, wortmannin, reduced mechanical hyperalgesia induced by KO_2 administration in the ipsilateral paw (Panel A). The i.t. injection did not alter the hyperalgesia in the contra-lateral (Panel B) paw. Results are presented as means ± s.e.m. of 6 mice per group per experiment, and are representative of 2 separated experiments. **P*<0.05 compared to the saline group; #*P*<0.05 compared to the KO_2 group; ***P*<0.05 compared to the doses of 0.3 and 1 μg of the inhibitor tested (One-way ANOVA followed by Bonferroni's test).

Spinal MAP kinase ERK has an important participation in KO_2-induced mechanical hyperalgesia

The dose of 1 μg was not capable of alter KO_2-induced mechanical hyperalgesia (Fig. 2A). However, the dose of 3 μg of PD98059 was capable of inhibit the KO_2-induced hyperalgesia

starting 1 hour after stimulus and remaining until the last time point of analysis (Fig. 2A). The highest dose (10 μg) of PD98059 inhibited the mechanical hyperalgesia 1-7 h after stimulus injection (Fig. 2A). Also, the treatment i.t. with PD98059 did not alter the hyperalgesia in contra-lateral paw (Fig. 2B).

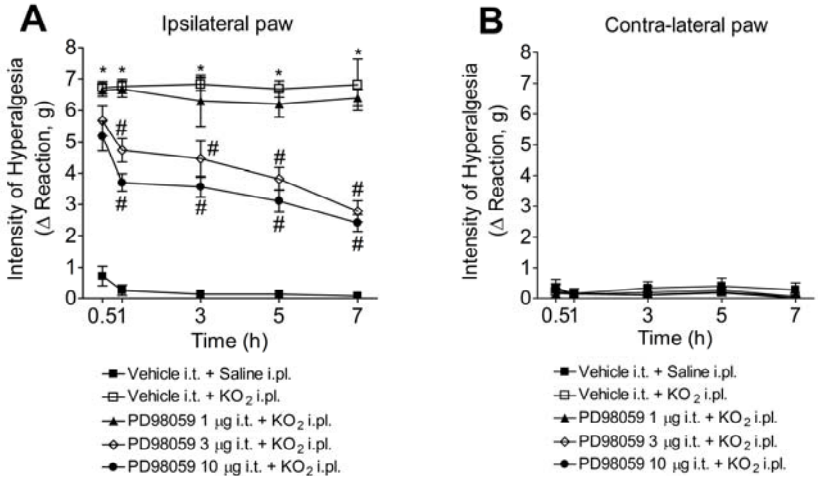


Figure 2. Spinal MAP kinase ERK has an important participation in KO_2-induced mechanical hyperalgesia. The i.t. administration of PD98059 inhibited the KO_2-induced mechanical hyperalgesia in the ipslateral paw (Panel A) and did not alter the hyperalgesia in the contra-lateral paw (Panel B). Results are presented as means ± s.e.m. of 6 mice per group per experiment, and are representative of 2 separated experiments. **P*<0.05 compared to the saline group; #*P*<0.05 compared to the KO_2 group (One-way ANOVA followed by Bonferroni's test).

Role of spinal MAP kinase p38 in KO_2-induced hyperalgesia

The lowest dose of SB202190 did not alter the KO_2-induced hyperalgesia in the ipsilateral paw (Fig. 3A). On the other hand, the doses of 3 and 10 μg i.t. of SB202190 inhibited the KO_2-induced hyperalgesia 30 min-7 h after stimulus injection (Fig. 3A). The SB202190 i.t. administration did not alter the mechanical hyperalgesia in the contra-lateral paw (Fig. 3B).

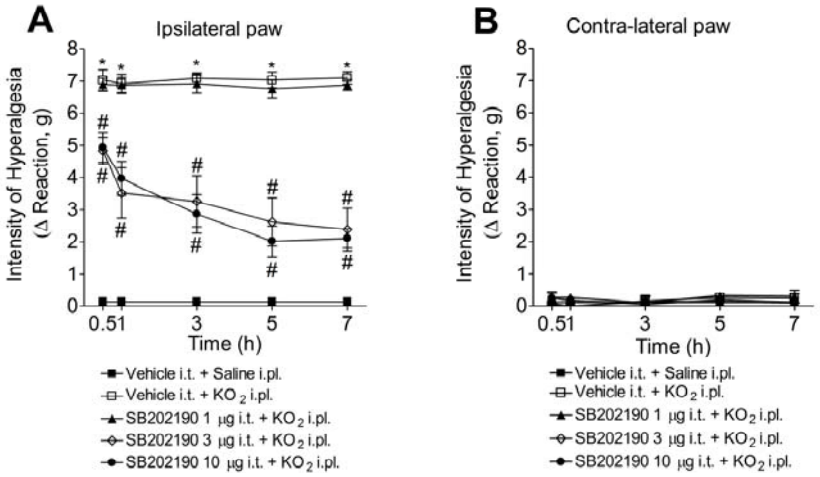


Figure 3. Role of spinal MAP kinase p38 in KO_2-induced hyperalgesia. Intrathecal treatment with the p38 inhibitor, SB202190, reduced the mechanical hyperalgesia induced by intraplantar (i.pl.) administration of the KO_2 stimulus in the ipsilateral paw (Panel A) and did not affect the contra-lateral paw (Panel B). Results are presented as means ± s.e.m. of 6 mice per group per experiment, and are representative of 2 separated experiments. **P*<0.05 compared to the saline group; #*P*<0.05 compared to the KO_2 group (One-way ANOVA followed by Bonferroni's test).

Role of spinal MAP kinase JNK in KO_2-induced hyperalgesia

The dose of 1 μg of SP600125 did not inhibit the KO_2-induced hyperalgesia (Fig. 4A). However the dose of 3 μg significantly inhibited the hyperalgesia 30 min-7 h after the i.pl. administration of the stimulus (Fig. 4A). The dose of 10 μg of SP600125 inhibited KO_2 hyperalgesia when compared to vehicle i.t. + KO_2 i.pl. and SP600125 1 μg i.t. + KO_2 i.pl. groups 30 min-7 h after stimulus, also this dose inhibited KO_2 hyperalgesia when compared to SP600125 3 μg i.t. + KO_2 i.pl. groups at 30 min, and 3-7 h after KO_2 injection (Fig. 4A). Furthermore, i.t. injection of SP600125 did not alter the hyperalgesia in the contra-lateral paw (Fig. 4B).

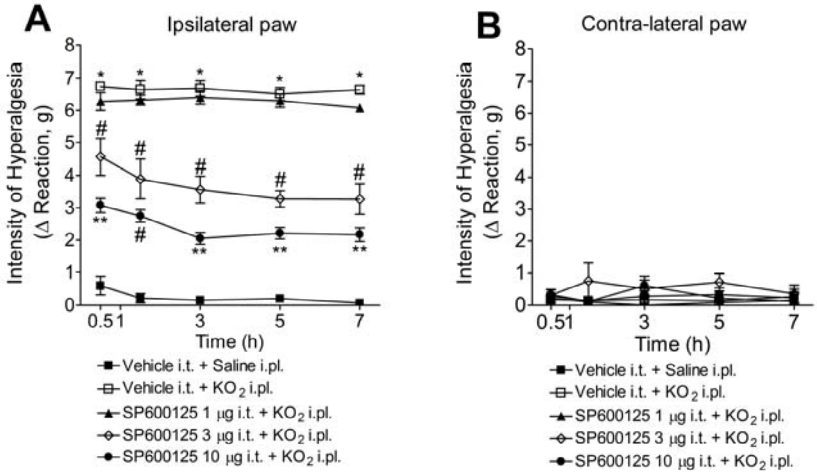


Figure 4. Role of spinal MAP kinase JNK in KO_2-induced hyperalgesia. The i.t. administration of SP600125 inhibited the KO_2-induced mechanical hyperalgesia in the ipsilateral paw (Panel A) but not alter the hyperalgesia in the contra-lateral paw (Panel B). Results are presented as means ± s.e.m. of 6 mice per group per experiment, and are representative of 2 separated experiments. **P*<0.05 compared to the saline group; #*P*<0.05 compared the KO_2 group; ***P*<0.05 compared to the doses of 1 and 3 μg of the inhibitor tested (One-way ANOVA followed by Bonferroni's test).

Combined Treatment with PI_3K and MAP kinases inhibitors at doses that are ineffective as single treatment reduces KO_2-induced hyperalgesia

Mice were treated i.t. with of PD98059 (1 μg), SB202190 (1 μg), SP600125 (1 μg), 0.3 μg wortmannin (a dose that *per se* has no effect), or co-treatment with 1 μg of each MAP kinase inhibitors (PD98059, SP600125, SB202190) plus 0.3 μg of wortmannin in a single injection or vehicle (5 μl of 20% DMSO in saline) 30 min before KO_2 (Fig. 5) stimulus. The KO_2 injection induced mechanical hyperalgesia 30 min-7h that was not reversed only by the treatment of each of MAP kinase or PI_3K inhibitors separated, doses that were inefficient as single treatment in the first set of experiments (Fig. 5A). However, when we put those inhibitors together into a co-treatment, it significantly inhibited the KO_2-induced hyperalgesia 30 min-7h (Fig. 5A). Also, i.t. injection of co-treatment did not alter the hyperalgesia in the contra-lateral paw (Fig. 5B).

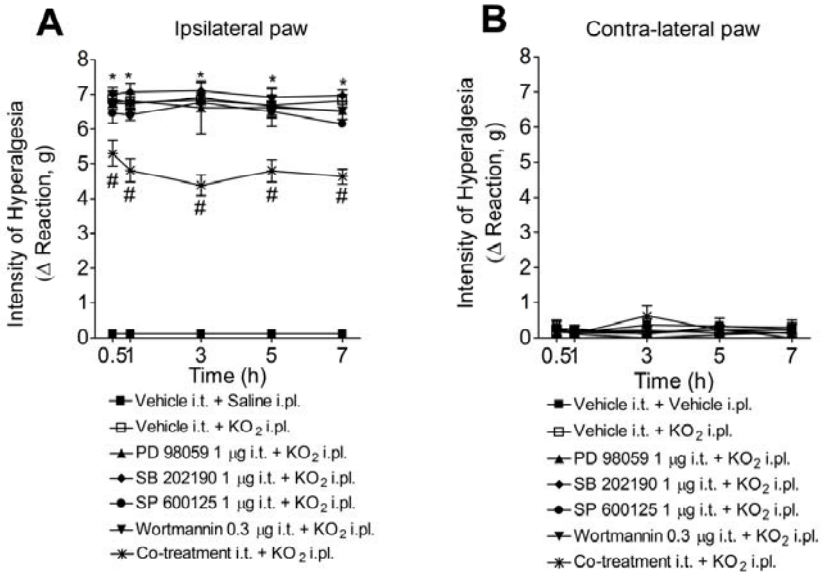


Figure 5. Combined Treatment with PI_3K and MAP kinases inhibitors at doses that are ineffective *per se* reduces KO_2-induced hyperalgesia. Mice received an i.t. pretreatment with of PD98059 (1 μg), SB202190 (1 μg), SP600125 (1 μg), wortmannin (0.3 μg), or co-treatment with 1 μg of each MAP kinase inhibitors (PD98059, SP600125, SB202190) plus 0.3 μg of wortmannin in a single injection or vehicle before KO_2 stimulus. The intensity of the mechanical hyperalgesia was evaluated in the ipsilateral (Panel A) and contra-lateral (Panel B) paws. Results are presented as means ± s.e.m. of 6 mice per group per experiment, and are representative of 2 separated experiments. **P*<0.05 compared to the saline group; #*P*<0.05 compared to the KO_2 group (One-way ANOVA followed by Bonferroni's test).

Role of spinal microglia in KO_2-induced hyperalgesia

The lower dose of minocycline (15 μg i.t.) was capable of inhibiting the mechanical hyperalgesia 3-5h after the injection of KO_2 stimulus (Fig. 6A). On the other hand, the treatment i.t. with minocycline in the doses of 50 and 150 μg inhibited the KO_2-induced hyperalgesia 30 min-7h after stimulation (6A). Furthermore, i.t. treatment with minocycline did not alter the hyperalgesia in the contra-lateral paw (Fig. 6B).

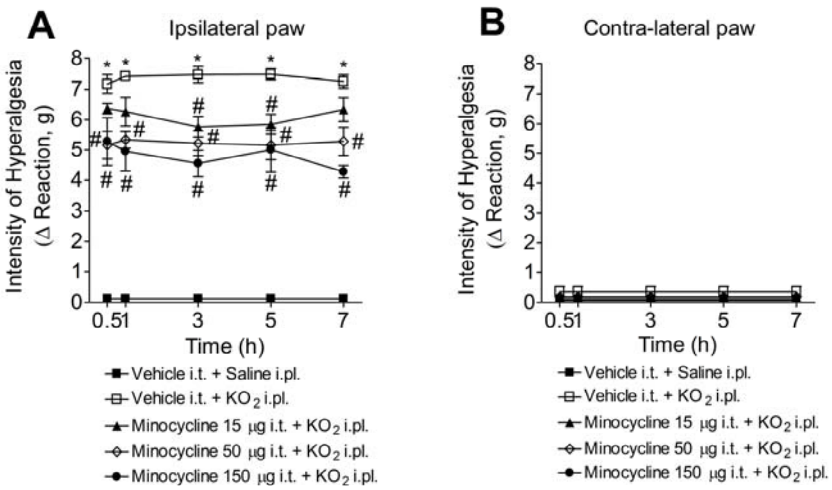


Figure 6. Role of spinal microglia in KO_2-induced hyperalgesia. The i.t. treatment with microglia inhibitor, minocycline, inhibited the mechanical hyperalgesia induced by i.pl. administration of KO_2 in the ipsilateral paw (Panel A). The i.t. injection did not affect the contra-lateral paw (Panel B). Results are presented as means ± s.e.m. of 6 mice per group per experiment, and are representative of 2 separated experiments. **P*<0.05 compared to the saline group; #*P*<0.05 compared to the KO_2 group (One-way ANOVA followed by Bonferroni's test).

Role of spinal astrocytes in KO_2-induced hyperalgesia

The dose of 0.05 μg i.t. of fluorocitrate did not inhibit the mechanical hyperalgesia induced by KO_2 stimulus (Fig. 7A). On the other hand, i.t. treatment with 0.15 μg of fluorocitrate significantly inhibited the mechanical hyperalgesia induced by i.pl. administration of KO_2 1-7 h after stimulation (Fig. 7A). Furthermore, the dose of 0.45 μg i.t. of fluorocitrate produced a significantly inhibition of KO_2-induced hyperalgesia compared to vehicle i.t. + KO_2 i.pl. group in all evaluation times after KO_2 administration, and compared to fluorocitrate 0.05 μg i.t. + KO_2 i.pl. and fluorocitrate 0.15 μg i.t. + KO_2 i.pl. groups 30 min-1h after i.pl. stimulation (Fig. 7A). The treatment i.t. with fluorocitrate did not alter the hyperalgesia in the contra-lateral paw (Fig. 7B).

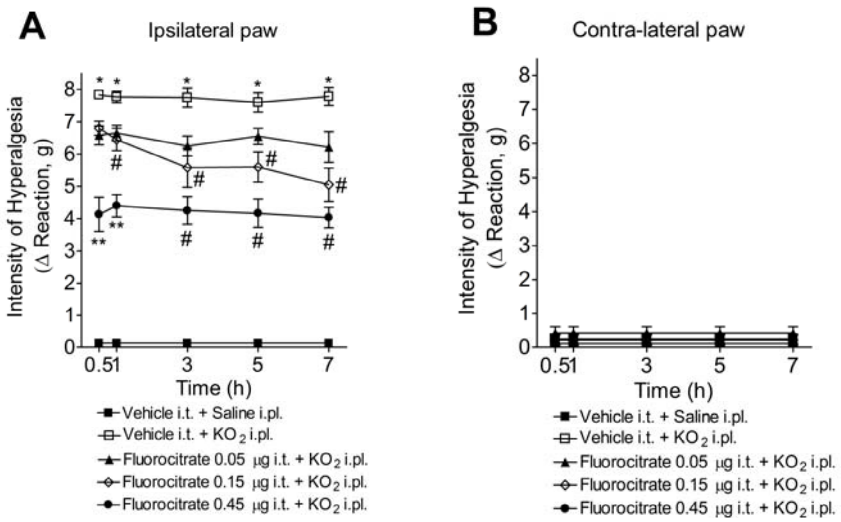


Figure 7. Role of spinal astrocytes in KO_2-induced hyperalgesia. Fluorocitrate i.t. injection reduced the KO_2-induced mechanical hyperalgesia in the ipsilateral paw (Panel A) and the treatment did not alter the hyperalgesia in the contra-lateral paw (Panel B). Results are presented as means ± s.e.m. of 6 mice per group per experiment, and are representative of 2 separated experiments. **P*<0.05 compared to the saline group; #*P*<0.05 compared to the KO_2 group; ***P*<0.05 compared to the doses of 0.05 and 0.15 µg of the inhibitor tested (One-way ANOVA followed by Bonferroni's test).

Role of spinal PI_3K, MAP kinases (ERK, p38, and JNK), microglia and astrocytes in KO_2-induced abdominal contortions

The doses of PI_3K, MAP kinases (PD98059, SB202190 or SP600125), microglia, or astrocyte inhibitors that were significantly efficient in the experiments of mechanical hyperalgesia were used to evaluate the participation of these spinal signalization pathways in the writhing response test (Fig. 8). For that, mice were treated with wortmannin (3 µg), PD98059 (3 µg), SB202190 (3 µg), SP600125 (3 µg), or vehicle (5 µL of 20 % DMSO in Saline) (Fig. 8A) and, with minocycline (50 µg), fluorocitrate (0.45 µg) or vehicle (5 µL of 2 % DMSO in Saline) 30 min before intraperitoneal (i.p.) stimulus of KO_2 (1 mg/animal) (Fig. 8B). KO_2 i.p. injection induced significant overt pain-like behavior response that was reversed by the i.t. treatment with wortmannin, PD98059, SB202190, or SP600125 (Fig. 8A). Furthermore, i.t. treatment with glial cells inhibitors, minocycline or fluorocitrate, reduced the writhing response induced by the i.p. injection of KO_2 (Fig. 8B).

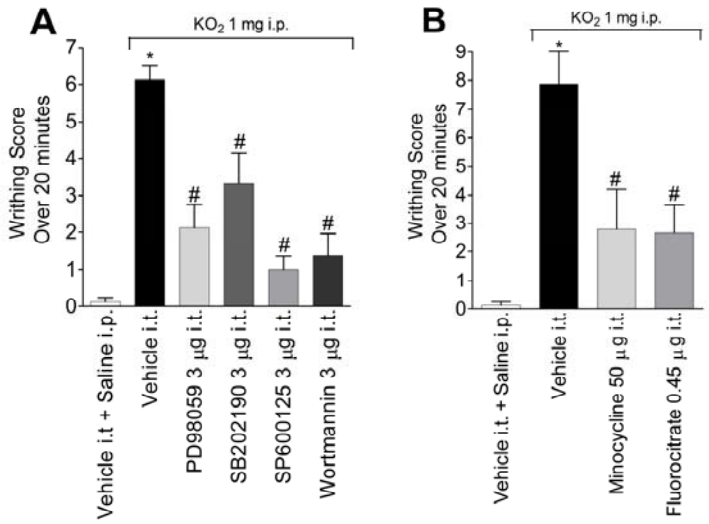


Figure 8. Role of spinal PI_3K, MAP kinases (ERK, p38, and JNK), microglia and astrocytes in KO_2-induced abdominal contortions. Mice were pretreated with PI_3K inhibitor (wortmannin), MAP kinase inhibitors (PD98059, SB202190 or SP600125), or vehicle intrathecally, 30 min before KO_2 injection (1 mg/animal, i.p.) (Panel A); or treated i.t. with minocycline and fluorocitrate, or vehicle 30 min before KO_2 injection (Panel B). The cumulative number of abdominal contortions (writhing score) was determined over 20 min. Results are presented as means ± s.e.m. of 6 mice per group per experiment, and are representative of 2 separated experiments. **P*<0.05 compared to the saline group; #*P*<0.05 compared to the KO_2 group (One-way ANOVA followed by Bonferroni's test).

Discussion

Superoxide anion has been postulated as an important mediator of pain in inflammatory, neoplastic and neuropathic conditions. Using a model of superoxide donor injection (KO_2 - potassium superoxide) in which superoxide induces inflammation (paw edema, leukocyte recruitment, overt pain-like behavior and hyperalgesia) and oxidative stress (Maioli NA et al., under preparation), it was demonstrated that peripheral superoxide induces mechanical hyperalgesia and abdominal contortions by spinal activation of PI_3K, MAP kinases and glial cells.

Superoxide anion mediates inflammatory hyperalgesia by inducing events such as neutrophil recruitment, stimulating the release of various cytokines such as TNF-α and IL-1β, reacting with nitric oxide to form peroxynitrite, and activating poly-ADP-ribose-polymerase [26,31,33]. These conclusions were draw based on approaches to modulate the levels of superoxide anion. In the present model, it seems that the nociceptive effects of potassium superoxide are indirect since depend on the peripheral (site of stimulus injection) production of additional nociceptive molecules including the cytokine TNF-α (Yamacita-Borin FY et al., unpublished data) and the peptide endothelin-1 (ET-1) (Serafim KGG et al., unpublished data). In these studies it was observed that administration of potassium superoxide-induced overt-pain like behavior, hyperalgesia, paw edema, neutrophils and macrophages recruitment and oxidative stress that was reversed by targeting TNF-α acting in TNFR1 receptor (tumor necrosis factor receptor type 1) through using TNFR1 knockout mice and soluble TNF-α receptor (Etanercept) (Yamacita-Borin FY et al., unpublished data), ET_A (Clazosentan) and ET_B (PQ-788), and a mixed (antagonist of both ET_A and ET_B receptors - Bosentan) endothelin receptor antagonists (Serafim KGG et al., unpublished data), morphine and quercetin (Maioli NA et al., under preparation). Yamacita-Borin FY et al. (unpublished data) also demonstrate that TNF-α-induced mechanical hyperalgesia was reduced by treatment with apocynin (a NADPH oxidase complex inhibitor) and tempol (a SOD mimetic) suggesting that TNF-α also acts by inducing superoxide production.

The mediation of superoxide-induced nociception by TNF-α and ET-1 is in accordance with the present data on PI_3K, MAP kinase and glial cells since there is evidence that TNF-α and ET-1 induce hyperalgesia via PI_3K, MAP kinase and glial cells activation [2,43-45]. For instance, TNF-α-induced spinal long-term potentiation was completely blocked by pretreatment with p38 and JNK inhibitors [45] and ET-1-induced mechanical hyperalgesia in rats was reduced by the inhibitors of p38, ERK1/2, and JNK [44].

One important perspective shown herein is that the co-treatment with doses of kinase inhibitors at doses that are ineffective as single treatment significantly reduced superoxide-induced mechanical hyperalgesia. Therefore, such kind of multiple drug treatment could reduce the dose of each drug and possibly the adverse side effects. In the case of PI_3K and MAP kinases, this better profile obtained by co-treatment is also related to the fact that PI_3K may activate MAP kinases and that each MAP kinase may also trigger different signaling pathways and transcription factors. For instance, PI_3K activates ERK in spinal neurons following intraplantar formalin injection [8] and after capsaicin and NGF stimulation in primary sensory neurons [36]. Activation of p38 activates the transcription factor NFκB, and ERK and JNK activate protein-1 (AP-1) [46-50].

Although we did not address the cells in which each kinase is activated during superoxide-induced pain, taking into account other models of inflammatory pain, we can speculate a sequential activation of PI_3K and MAP kinases in different cells in the spinal cord. After inflammatory stimulus PI_3K can be activated in the spinal cord neurons and then it is responsible for ERK activation, possibly in neurons [8-10,36,51,52]. In turn, JNK and p38 activation may occur in microglia, astrocytes and neurons [10,23,53]. This evidence also emphasizes the importance of spinal microglia and astrocytes in the development of inflammatory, neoplastic and neuropathic pain states. Therefore, the present demonstration that peripheral superoxide administration activates spinal glial cells further contributes to the notion that targeting the activity of glial cells has therapeutic potential.

Therefore, it is consistent among diverse models that pain depends on spinal activation of MAP kinases, PI_3K and glial cells, which are potential spinal targets for pain control. Peripheral administration of superoxide induces the spinal activation of PI_3K, MAP kinases and glial cells. The combined treatment with inhibitors of PI_3K and MAP kinases at doses that are ineffective as single treatment were effective in reducing superoxide hyperalgesia suggesting that combined treatment could be an approach to reduce the dose of these inhibitors to control peripheral superoxide-mediated inflammatory pain.

Acknowledgments

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5 CONCLUSÃO

Nesse estudo foi demonstrado que as vias de sinalização PI_3K, ERK, p38, JNK e células, microglia e astrócitos, espinais contribuem para a dor inflamatória visualizada através de hiperalgesia e contorções abdominais induzidas pelo superóxido de potássio, um doador de ânion superóxido. Por meio da utilização de inibidores específicos para cada alvo do estudo, foi possível ainda observar que pode existir ativação sinérgica/sequencial destas vias neste modelo. Assim, este trabalho demonstra um avanço no entendimento da dor inflamatória decorrente do aumento da geração do ânion superóxido. Este entedimento permite que o modelo seja utilizado com maior segurança para a busca de novas terapias que sejam mais eficazes no tratamento da dor inflamatória e com menor incidência de eventos adversos, diferentemente dos tratamentos utilizados atualmente.

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ANEXOS

ANEXO A
Normas da revista Free Radical Biology & Medicine

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Chapter in edited book:

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