FELIPE ALMEIDA DE PINHO RIBEIRO

DOR E INFLAMAÇÃO INDUZIDAS PELO KO_2:

PARTICIPAÇÃO DO FATOR DE TRANSCRIÇÃO NF-κB

Londrina
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Dissertação de mestrado apresentada ao curso de Patologia Experimental da Universidade Estadual de Londrina, como requisito parcial à obtenção do título de Mestre em Patologia Experimental.

Orientador: Prof. Dr. Waldiceu Aparecido Verri Júnior

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RESUMO

O processo inflamatório produz grandes quantidades de ânion superóxido $O_2^{\bullet-}$ que, por sua vez, sensibilizam as fibras nociceptivas e levam aos processos patológicos de dor, como a hiperalgesia e a alodinia. Contudo, a participação do $O_2^{\bullet-}$ nos mecanismos fisiopatológicos da dor ainda estão pouco esclarecidos. O presente estudo investigou a participação do fator nuclear *kappa* B (NF-κB), um fator de transcrição estreitamente relacionado com os efeitos deletérios da inflamação, na dor, na hiperalgesia, e no estresse oxidativo induzidos pela injeção de solução contendo superóxido de potássio (KO_2), um doador de ânion superóxido. Para isso, camundongos Swiss foram tratados com pirrolodina ditiocarbamato (PDTC, 3 – 100 mg/kg, 100 µL, subcutâneo), um inibidor do NF-κB, 1 h antes da injeção intraplantar ou intraperitoneal de KO_2. O tratamento com PDTC reduziu de maneira dose dependente a hiperalgesia aos estímulos mecânicos e térmicos, a formação do edema, e o recrutamento de neutrófilos e macrófagos no local do estímulo. Além disso, o tratamento com PDTC também reduziu os comportamentos de dor (contorções abdominais, sacudidas de pata, e lambidas na pata) induzidos pelo KO_2. Conforme os parâmetros avaliados, o tratamento com PDTC exibiu efeitos protetores no local do estímulo por reduzir o recrutamento de monócitos e de neutrófilos, a depleção de glutationa reduzida (GSH), e a produção de citocinas inflamatórias (IL-1β, TNF-α, and IL-10). Na medula espinal (L4 – L5), o tratamento com PDTC também inibiu as alterações dos níveis de citocinas e de GSH induzidas após o estímulo periférico com KO_2. A atividade da via do NF-κB também foi avaliada no local do estímulo. O tratamento com PDTC reduziu a degradação do inibidor do NF-κB alfa (IκBα), um indicador da atividade do NF-κB, induzida pelo KO_2 nos intervalos de 1 e 3 h após o estímulo, mas não no intervalo de 0.5 h. Dessa forma, o presente estudo demonstrou que o KO_2 induz a dor e a inflamação através do aumento do estresse oxidativo, da produção de citocinas, e da ativação do NF-κB, de modo sensível ao tratamento com PDTC.

Palavras-chave: Superóxido de potássio. Pirrolidina ditiocarbamato. Inflamação. Dor. NF-κB.

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ABSTRACT

The inflammatory process produces large amounts of superoxide anion ($O_2^{\bullet-}$) which, in turn, sensitize nociceptive fibers leading to the pathological processes of pain such as hyperalgesia and allodynia. However, the role of $O_2^{\bullet-}$ in the physiopathological mechanisms of pain are still poorly understood. The present study investigated the role of nuclear factor *kappa* B (NF-κB), a transcription factor that is related to the deleterious effects of inflammation, in pain, hyperalgesia, and oxidative stress induced by potassium superoxide (KO_2), a superoxide anion donor. Swiss mice were treated with pyrrolidine dithiocarbamate (PDTC 3 - 100 mg/kg , 100 mL , subcutaneous), an inhibitor of NF-κB, 1 h before the intraplantar or intraperitoneal injection of KO_2. Treatment with PDTC reduced hyperalgesia to mechanical and thermal stimuli, edema formation, and recruitment of neutrophils and macrophages at the site of stimulation in a dose dependent manner. Furthermore, treatment with PDTC also reduced KO_2-induced pain-like behaviors (abdominal writhing, paw flinching, and paw licking). According to the parameters employed in this study, the treatment with PDTC exhibited protective effects at the site of stimuli by reducing monocyte and neutrophil recruitment, depletion of reduced glutathione (GSH), and production of inflammatory cytokines (IL-1β, TNF-α, and IL-10). At the spinal cord level (L4 - L5), PDTC also inhibited the modulation of cytokine and GSH levels induced by peripheral stimulus with KO_2. Moreover, the NF-κB pathway activity was evaluated at the site of stimulus injection. Treatment with PDTC reduced the KO_2-induced degradation of NF-κB inhibitor *alpha* (IκBα), an indicator of NF-κB activation, at 1 and 3 h after stimulus injection, but not at 0.5 h. Thus, the present study demonstrated that KO_2 induces pain and inflammation by increasing oxidative stress, cytokine production, and activation of NF-kB in a PDTC-sensitive manner.

Keywords: Potassium superoxide. Pyrrolidine dithiocarbamate. Inflammation. Pain, NF-κB.

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COX	Ciclo-oxigenase
CpG-DNA	Citosina seguida de guanina
CTL	Receptores semelhantes a lectina tipo C
DAMP	Padrões moleculares associados a danos
DRG	Gânglio da raiz dorsal
dsRNA	RNA fita dupla
GSH	Glutationa reduzida
HMGB1	Proteína do grupo de alta mobilidade B1
$HO^{\bullet}$	Radical hidroxil
HSP	Proteína de choque térmico
i.p	Intraperitoneal
i.pl	Intraplantar
ICAM	Molécula de Adesão Intracelular
iE-DAP	Ácido γ-D-glutamil-meso-diaminopimélico
IL	Interleucina
iNOS	Óxido nítrico sintase induzível
KO_2	Superóxido de potássio
LPS	Lipopolissacarídeo
LPS	Lipopolissacarídeo
MDP	Muramil dipeptídeo
MPO	Mieloperoxidase
mRNA	RNA mensageiro
MSU	Cristais de urato monossódico
NADPH	Fosfato de dinucleotídio de nicotinamida e adenina
NF-κB	Fator nuclear kappa B
NLR	Receptor semelhante a NOD
NO	Óxido nítrico
NOD	Domínio de oligomerização e ligação de nucleotídeos
$O_2^{\bullet-}$	Radical ânion superóxido
$ONOO^-$	Peroxinitrito
PAMP	Padrões moleculares associados a patógenos
PGE_2	Prostaglandina E_2

PRR	Receptores de reconhecimento de padrões
RIG	Gene induzido pelo ácido retinóico
RLR	Receptores semelhantes a RIG
ROI	Intermediários reativos de oxigênio
ROS	Espécies reativas de oxigênio
SAP130	Subunidade da histona deacetilase
SNC	Sistema nervoso central
SOD	Superóxido dismutase
ssRNA	RNA fita simples.
TLR	Receptores semelhantes ao Toll
TNF	Fator de Necrose Tumoral
VCAM	Molécula de Adesão Vascular

SUMÁRIO

1 INTRODUÇÃO

As situações adversas impostas pelo ambiente geram estresse e desequilíbrio ao organismo e, dessa forma, impõem ao organismo a necessidade de reações benéficas e adaptativas. Dois principais sistemas altamente adaptativos, ou seja, capazes de interpretar e responder de maneira positiva a uma grande diversidade de estímulos, são os sistemas nervoso e imunológico. As situações que apresentam potencial lesivo ao organismo e, por esse motivo, ameaçam a sua integridade e sobrevivência, retém atenção especial de ambos os sistemas. Neste sentido, a dor, uma resposta adaptativa desempenhada pelo sistema nervoso, e a inflamação, resposta integrada do tecido afetado com o sistema imunológico, são as duas primeiras linhas de defesa do organismo e agem de forma integrada para a restauração do equilíbrio. Porém, em alguns casos essa integração pode ocorrer de maneira mal-adaptativa e contribuir com o desenvolvimento das doenças.

1.1 DOR

A capacidade de detectar estímulos nocivos é crucial à manutenção da integridade do organismo e, consequentemente, à sua sobrevivência. Nesse sentido, a experiência da dor não é por acaso relatada como desagradável. O comitê de taxonomia da 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” (INTERNATIONAL ASSOCIATION FOR THE STUDY OF PAIN, 1994). A importância da dor é exemplificada pela observação de indivíduos incapazes de identificar estímulos nocivos provenientes de objetos pontiagudos, de fontes de calor, ou mesmo de danos internos como uma fratura óssea. A ausência de dor nesses indivíduos impede a indução de comportamentos adequados que levam, por exemplo, à conservação da região afetada, ou mesmo à aquisição da memória aversiva à situação de risco (BUTLER; FLEMING; WEBB, 2006). Por outro lado, as alterações nos componentes que levam à experiência da dor podem exacerbar a intensidade dos estímulos nocivos (hiperalgesia) ou mesmo fazer com que esta ocorra na presença de estímulos inócuos (alodinia). A exacerbação ou persistência da experiência da dor está presente em diversas doenças e representa a principal causa da procura por atendimento médico. Apesar de a dor fisiológica ser um

mecanismo de auto-conservação indispensável, a dor persistente e exagerada reduz drasticamente a qualidade de vida e gera incapacitação dos indivíduos, sendo considerada uma doença e não apenas um sintoma. Recentemente, as bases que levam à experiência da dor e, mais especificamente, as bases celulares e moleculares da nocicepção, ou seja, da capacidade em detectar e transmitir os estímulos nocivos até os centros superiores do sistema nervoso, têm sido evidenciadas pela utilização de fármacos experimentais em estudos *in vitro* e *in vivo* (WANG; WANG, 2003).

1.1.1 Fisiologia da Nocicepção

Dá-se o nome de nocicepção ao processo de detecção e transmissão da informação proveniente de um estímulo nocivo (alta intensidade). Este processo tem como objetivo principal identificar rapidamente as situações que representam risco à integridade do organismo, induzindo alterações fisiológicas e comportamentais que impeçam a exposição a essas situações ou então, no caso de uma injúria ocorrida, que favoreçam a conservação e recuperação do indivíduo. Entre os componentes que auxiliam no processo de nocicepção, os neurônios nociceptivos, integrantes do sistema nervoso somatossensorial, são componentes fundamentais em todas as etapas. Os neurônios nociceptivos de primeira ordem contém receptores de membrana em suas terminações nervosas que são especializados na detecção de estímulos de alta intensidade de natureza mecânica, térmica ou química. Os corpos celulares dos neurônios nociceptivos aferentes primários localizam-se no gânglio da raiz dorsal (DRG) ou no gânglio trigeminal. As fibras nociceptivas constituídas por neurônios nociceptivos de primeira ordem encontram-se amplamente distribuídas nos diferentes tecidos e podem ser classificadas nos subtipos A-δ (mielinizadas, de transmissão rápida - cerca de 12 a 30 m/s) e C (não mielinizadas, de transmissão lenta - cerca de 0,5 a 2 m/s). De modo geral, estímulos provenientes das fibras Aδ provocam dor acentuada (em “picada”), enquanto que aqueles provenientes das fibras C provocam dor latente e relativamente dispersa (em “queimação”) (BASBAUM et al., 2009). Assim como ocorre com outras fibras somatossensoriais, os estímulos nocivos agem através dos receptores dos neurônios nociceptivos de primeira ordem, induzindo alterações na permeabilidade da membrana plasmática neuronal, no fluxo iônico e no potencial da membrana. Dois tipos principais de receptores podem gerar essas alterações de permeabilidade neuronal nas fibras nociceptivas – os receptores ionotrópicos e os receptores metabotrópicos (BASBAUM et al., 2009).

Quando as alterações na permeabilidade da membrana neuronal levam à despolarização, ou seja, modificam as cargas iônicas da fibra nociceptiva em uma intensidade que ultrapassa o chamado limiar de ativação, ocorre transmissão do estímulo ao longo de toda a extensão do neurônio nociceptivo (Figura 1). Após ser gerado perifericamente por um estímulo nociceptivo, o impulso percorre o neurônio nociceptivo de primeira ordem até o corno dorsal da medula espinal, ou até o núcleo trigeminal, onde estes neurônios fazem sinapses com as terminações dos neurônios nociceptivos de segunda ordem. A informação é então transmitida ao neurônio nociceptivo de segunda ordem por neurotransmissores excitatórios (como o glutamato e a substância P) que são liberados pelo neurônio nociceptivo primário na fenda sináptica. De maneira semelhante à ativação periférica, estímulos com intensidade suficiente para despolarizar a membrana do neurônio nociceptivo de segunda ordem são conduzidos até os centros superiores (supraespinais) localizados no encéfalo. Estes estímulos são então analisados e interpretados como a experiência de dor (Figura 1) (BASBAUM et al., 2009). Uma vez que as bases da nocicepção encontram-se presentes tanto nos processos fisiológicos como nas doenças, a modulação desta última (principalmente farmacológica) requer conhecimento aprofundado dos mecanismos que diferenciam os dois estados a fim de desenvolver abordagens terapêuticas eficazes para as situações de doença sem que a sua atividade fisiológica, essencial à integridade do organismo, seja alterada.

Com exceção de alguns casos, as alterações que levam à dor sem função adaptativa (desnecessária, persistente, e/ou exacerbada) têm em comum a participação de células e mediadores da resposta inflamatória. Desse modo, serão consideradas aqui apenas as alterações da nocicepção que ocorrem no contexto da inflamação, ou seja, da dor inflamatória persistente.

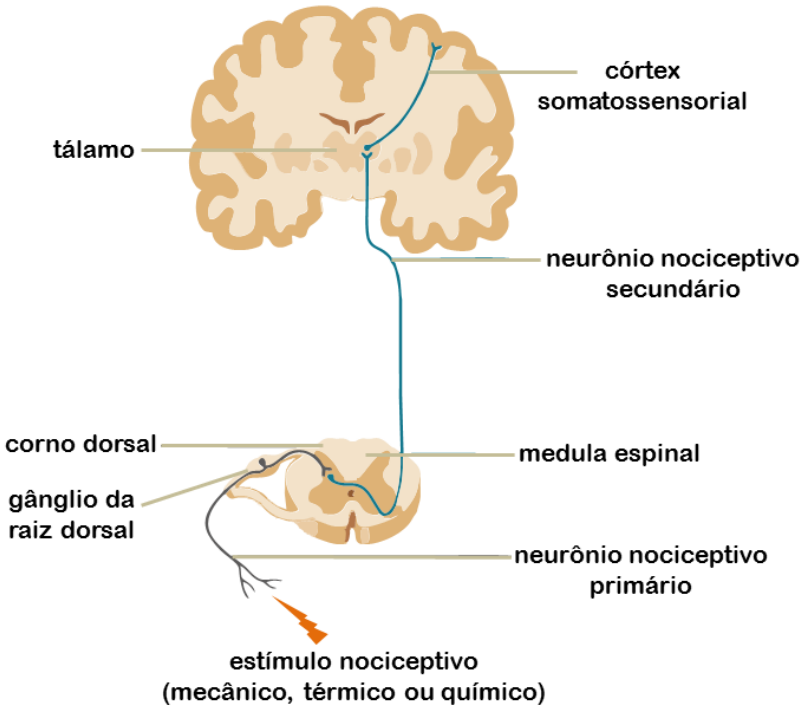

Figura 1 – Transmissão da informação nociceptiva. Os neurônios nociceptivos primários conduzem a informação de um estímulo nociceptivo, passando pelo gânglio da raiz dorsal, até o corno dorsal da medula espinal, onde fazem sinapse com neurônios nociceptivos secundários. Estes, por sua vez, levam a informação nociceptiva até os centros superiores do SNC, como tálamo e córtex somatossensorial, onde a informação é integrada (intensidade, localização) e interpretada como dor (figura adaptada de BASBAUM et al., 2009).

1.2 Inflamação Aguda

Mesmo dispondo da capacidade de identificar estímulos nocivos e, dessa forma, evitar as situações de risco, é comum que o organismo se depare esporadicamente com danos de diversas intensidades. O dano tecidual, mesmo uma simples escoriação, permite que microrganismos tenham acesso ao tecido exposto. Em casos como este, onde o sistema nervoso é incapaz de impedir a progressão de um processo infeccioso, a imunidade inata, através da resposta inflamatória, passa a ser o componente responsável por esta proteção inicial e pela manutenção da integridade do organismo injuriado.

A reação inflamatória caracteriza-se por modificações dos componentes celulares e vasculares que ocorrem tanto no local do estímulo como de maneira sistêmica, e tem como sinais clínicos a presença de calor, rubor, edema, dor, e a perda de função (“trade-off”). É um processo ativo, pervasivo (recruta células de outros tecidos), e estreitamente regulado por

moléculas sinalizadoras, cuja função principal é eliminar, isolar, ou conter rapidamente a fonte do distúrbio, remover o tecido danificado, e restaurar a homeostase tecidual (MEDZHITOV, 2008; SOEHNLEIN; LINDBOM, 2010). A inflamação aguda pode ser dividida (didaticamente) em duas etapas: fase de ativação e fase efetora.

1.2.1 Fase de Ativação

A ativação da resposta inflamatória, assim como ocorre na nocicepção, depende de estímulos que sinalizam perigo à integridade do organismo. De maneira breve, a sinalização de um processo infeccioso e/ou de lesão tecidual induz, inicialmente, a ativação de células que se encontram próximas ao local do estímulo, como macrófagos, mastócitos, e células endoteliais. Essa ativação pode ocorrer por meio de uma grande quantidade de estímulos inflamatórios. Os estímulos cujos mecanismos de ativação e de transdução de sinal estão bem estabelecidos no contexto da resposta inflamatória incluem: os padrões moleculares associados a patógenos (PAMP – lipopolissacarídeo, ácido lipoteicóico, mananas), indicadores de um processo infeccioso; os padrões moleculares associados a danos (DAMP – ATP, catepsina B, peptídeo formilado, fragmentos de ácido hialurônico), moléculas que sinalizam morte celular não fisiológica, rompimento de organelas, ou degradação da matriz extracelular; ou distúrbios redox intra e extracelulares (consumo de GSH, efluxo de potássio, produção de ânion superóxido) (TAKEUCHI; AKIRA, 2010). Este reconhecimento ocorre em sua grande maioria através dos receptores (transmembrana ou citoplasmáticos) de reconhecimento de padrões (PRR).

Atualmente existem 4 classes bem caracterizadas de PRR: os receptores transmembrana semelhantes a Toll (TLR), os receptores transmembrana semelhantes a lectina tipo-C (CLR), os receptores citoplasmáticos semelhantes a RIG (RLR), e os receptores citoplasmáticos semelhantes a NOD (NLR)(KUMAGAI; AKIRA, 2010; TAKEUCHI; AKIRA, 2010). Uma relação dos padrões moleculares reconhecidos pelos PRR está descrita na Tabela 1. Depois que um estímulo inflamatório é reconhecido pelos PRR, uma série de vias de sinalização intracelulares é ativada e, consequentemente, diversas alterações ocorrem nas células estimuladas e também nas células adjacentes. O conjunto dessas alterações leva aos eventos celulares e vasculares clássicos da inflamação aguda e caracterizam a fase efetora da resposta inflamatória.

Tabela 1 – Receptores de reconhecimento de padrões (PRR): Localização celular e ligantes.

PRR	Localização	PAMP	DAMP
TLR			
TLR1	membrana plasmática	triacil-lipoproteína	β-defensina 3
TLR2	membrana plasmática	diacil-lipoproteína	HMGB-1, HSP60, HSP70, miosina cardíaca, cristais de urato monossódico,
TLR3	endolisossomo	dsRNA	mRNA
TLR4	membrana plasmática	LPS	HMGB-1, elastase neutrofílica, fibrinogênio, HSP90, LDL oxidado, lactoferrina, S100A8
TLR5	membrana plasmática		
TLR6	membrana plasmática	diacil-lipoproteína	veriscan
TLR7 (murino) TLR8 (humano)	endolisossomo	ssRNA	ssRNA
TLR9	endolisossomo	CpG-DNA	complexo IgG-cromatina, DNA
TLR10	endolisossomo		
TLR11	membrana plasmática	moléculas semelhantes à profilina	
CLR			
Dectina-1	membrana plasmática	β-glucana	
Dectina-2	membrana plasmática	β-glucana	
Mincle	membrana plasmática		SAP130
RLR			
RIG-1	citoplasma	dsRNA (curto)	
MDA5	citoplasma	dsRNA (longo)	
LGP2	citoplasma		
NLR			
NOD1	citoplasma	iE-DAP	
NOD2	citoplasma	MDP	
NLRP3	citoplasma	MDP, LPS	ROS, MSU, efluxo de K^+

1.2.2 Fase Efetora

Após a ocorrência de uma injúria periférica, de modo geral, a ativação dos PRR ocorre inicialmente em células próximas ao tecido afetado, principalmente em macrófagos, mastócitos, e células endoteliais. Logo em seguida ocorre também a ativação de PRR em neutrófilos que são recrutados em grande número durante a resposta inflamatória aguda (Figura 2). A partir deste momento, os neutrófilos passam a representar a maior fonte dos sinais e mediadores da inflamação aguda. A transdução dos sinais reconhecidos pelos PRR induz a ativação de vias de sinalização que, através de fatores de transcrição e de fosforilação enzimática, levam à transcrição, tradução e produção de mediadores da resposta inflamatória efetiva. Entre as vias de sinalização ativadas pelos PRR, muitas convergem na ativação do

fator de transcrição nuclear *kappa* B (NF-κB) e desempenham papel fundamental na inflamação (MCDONALD; BALD; CASSATELLA, 1997; MORGAN; LIU, 2011).

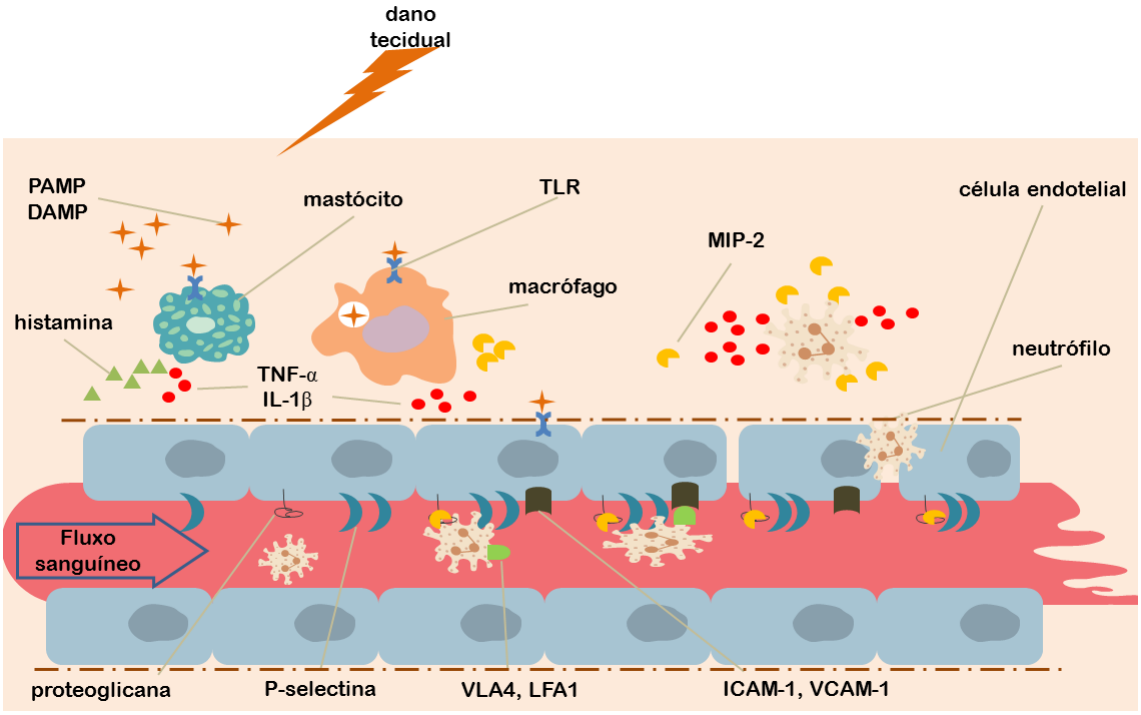

Figura 2 – Reação inflamatória aguda. A lesão ou infecção tecidual induz a reação inflamatória através de moléculas sinalizadoras de dano (DAMP) ou infecção (PAMP), respectivamente. Essas moléculas estimulam a produção de mediadores inflamatórios (histamina, TNF-α, IL-1β, IL-6) via receptores de reconhecimento de padrões (PRR) presentes nas células residentes do tecido afetado (macrófagos, mastócitos). Esses mediadores inflamatórios servem para a comunicação entre as células para o desenvolvimento da resposta inflamatória composta pelos sinais cardinais: dor, eritema, edema e aumento de temperatura. Dependendo da intensidade do processo inflamatório, há a perda de função no tecido afetado. Eventos primordiais na inflamação como o recrutamento celular também são modulados por mediadores espécies reativas.

1.2.2.1 *NF-κB*

Para que ocorra a produção de uma determinada proteína, é necessário que uma porção do DNA seja transcrita em RNA mensageiro pela enzima RNA polimerase II. A capacidade da RNA polimerase II em atuar de maneira específica nos genes correspondentes às proteínas de interesse para a célula e em um determinado momento depende de sua ligação às regiões promotoras destes genes. Para que esta ligação ocorra, é necessário que proteínas denominadas fatores de transcrição estejam ligadas aos elementos da região promotora. Classicamente, os fatores de transcrição possuem dois domínios funcionas, sendo um capaz de ligar à região promotora e outro à RNA polimerase II. Dessa forma, a atividade dos fatores

de transcrição permite que sinais celulares sejam convertidos em transcrição de genes específicos (LEMON; TJIAN, 2000).

A família NF-κB de fatores de transcrição é utilizada como modelo de transcrição induzível desde sua descoberta em 1986 (SEN; BALTIMORE, 1986). Em mamíferos, a família NF-κB é composta pelas seguintes subunidades: a p50, que provém do processamento da p105; a p52, originada do processamento de sua precursora p100; a p65, também descrita como RelA; a c-Rel e a RelB. Essas subunidades encontram-se no citoplasma como homo ou heterodímeros, inativados pela ligação dos inibidores do NF-κB (IκB) (OECKINGHAUS; HAYDEN; GHOSH, 2011). Assim, as regiões promotoras dos genes que deverão ser transcritos dependem da combinação formada entre os dímeros e também da degradação do IκB. O dímero mais comum e mais associado ao processo inflamatório é formado pelas subunidades p50 e p65. A ativação do heterodímero p50-p65 induz a resposta inflamatória durante infecções, lesão tecidual, e estresse oxidativo, e sua atividade pode ser controlada em diversos níveis. Como consequência, alterações na regulação da atividade de p50-p65 têm sido associadas às doenças inflamatórias, à autoimunidade, e ao câncer (OECKINGHAUS; HAYDEN; GHOSH, 2011). O IκBα bloqueia os sinais de localização nuclear do heterodímero p50-p65 e impede desta forma que ocorra a transcrição de genes relacionados à inflamação. Como citado anteriormente, a estimulação dos PRR que ocorre pelos sinais produzidos durante a lesão tecidual e/ou por indicadores de infecção leva à ativação do NF-κB através da degradação de IκBα. Desta forma, a redução das quantidades de IκBα representa um importante indicador da atividade desta via e, consequentemente, da inflamação. O complexo de quinases do IκB (IKK), formado pelas subunidades catalíticas IKKα, IKKβ, e pela subunidade regulatória IKKγ (NEMO), inicia o processo de marcação do IκBα através da fosforilação. O IκBα fosforilado é então ubiquitinado e degradado pelo proteossomo, permitindo assim que o heterodímero p50-p65 seja translocado até o núcleo da célula e induza a transcrição de genes inflamatórios (OECKINGHAUS; HAYDEN; GHOSH, 2011).

Os mediadores e sistemas enzimáticos que têm sua produção e atividade induzidas pela via do NF-κB durante o processo inflamatório agudo incluem: as citocinas inflamatórias fator de necrose tumoral alfa (TNF-α,), interleucina 1 beta (IL-1β), e IL-10 (anti-inflamatória); a forma precursora da endotelina 1 (ET-1) pré-pró-ET-1; as enzimas ciclooxigenase-2 (COX-2), óxido nítrico sintase induzivel (iNOS), e nicotinamida adenina dinucleotídeo fosfato oxidase (NADPH oxidase, ou NOX), moléculas de adesão (VCAM-1 e

ICAM-1) e quimiocinas (IL-8, ou proteína inflamatória de macrófagos – MIP-2) relacionadas à resposta inflamatória (TAKEUCHI; AKIRA, 2010).

Classicamente, os eventos vasculares e celulares da inflamação aguda são considerados consequências da síntese dos mediadores descritos acima (Figura 2). Citocinas (TNF-α e IL-1β), quimiocinas, prostanóides, leucotrienos e histamina, entre outros mediadores induzidos após ativação dos PRR, são capazes de induzir o aumento do aporte sanguíneo local, da permeabilidade vascular, e da exposição de moléculas de adesão pelas células endoteliais. Com o aumento no extravasamento de líquido circulante (transudato) há formação de edema no tecido adjacente e aumento de contato entre os leucócitos que escapam do fluxo laminar e o endotélio ativado (Figura 2). O endotélio ativado expõe moléculas de adesão leucocitária como selectinas P e E, responsáveis por interações de baixa intensidade com neutrófilos (rolamento) via PSGL-1 e selectina L, e as integrinas VCAM-1, ICAM-1 e PECAM, que se ligam com maior afinidade aos neutrófilos, via LFA1, VLA4 e Mac1, e favorecem a transmigração para o tecido (exsudato) (SADIK; KIM; LUSTER, 2011). A migração pelo tecido é então direcionada à favor do gradiente de concentração de moléculas quimiotáticas, como as quimiocinas (MIP-2) e os fragmentos do sistema complemento (C5a).

Durante o processo de migração, os mediadores quimiotáticos ativam gradualmente a liberação de grânulos pelos neutrófilos e a síntese de citocinas. Estes grânulos são classificados nos 4 tipos descritos a seguir na sua ordem de liberação: vesículas secretórias, contendo albumina e receptores de membrana, como integrinas e receptores de anticorpos (FcγRIII); grânulos terciários, contendo metaloproteases, gelatinase e leucolisina; grânulos secundários, contendo lactoferrina e lisozima; e os grânulos primários, contendo mieloperoxidases (MPO), elastase neutrofílica, catepsina G, defensinas, entre outras (SADIK; KIM; LUSTER, 2011). Inicialmente, ainda na etapa de rolamento, o contato com selectinas presentes no endotélio vascular permite que as vesículas secretórias sejam exportadas até a membrana do neutrófilo, aumentando assim a exposição das integrinas que favorecem a adesão firme e a migração. Os grânulos terciários contém as enzimas necessárias ao rompimento de fibras presentes na lâmina basal dos vasos e no tecido e favorecem dessa forma a transmigração e a migração pelo tecido. Por fim, a liberação dos conteúdos dos grânulos secundários e primários, com atividade microbicida, permite a destruição do patógeno.

Com a retirada do estímulo inicial, neutrófilos tendem a entrar em apoptose e são fagocitados pelos macrófagos, um processo chamado eferocitose. A eferocitose gera a mudança do fenótipo pró-inflamatório para um fenótipo pró-resolução nos macrófagos. Isso favorece a troca de classes dos mediadores sintetizados por estas células, levando ao aumento na produção dos chamados mediadores pró-resolução, como as maresinas e as lipoxinas (SERHAN, 2011). Em conjunto, estas e outras moléculas atuam na redução e no reparo dos danos provocados durante a inflamação aguda e promovem, dessa forma, a resolução (*catabasis*) do processo de forma ativa. Porém, falhas na etapa de resolução perpetuam a resposta inflamatória, um mecanismo responsável por diversas doenças. O estresse oxidativo ocorre quando há excesso na produção de espécies reativas e/ou depleção dos antioxidantes endógenos. Essa produção descompensada está presente em praticamente todas as doenças causadas por inflamação persistente e dessa forma também participa da dor inflamatória. De fato, há grande produção de espécies reativas durante a inflamação aguda e esta produção tem sido estreitamente relacionada com a destruição não só dos micro-organismos, mas também do tecido adjacente (HATTORI et al., 2010). É importante ressaltar que, durante o processo inflamatório persistente, há também sensibilização (periférica e central) de nociceptores e, consequentemente, dor inflamatória persistente. Dessa forma, a caracterização de compostos antioxidantes e a inclusão destes na terapia de doenças inflamatórias e da dor tem sido foco de muitos estudos. Tendo em vista que a grande maioria das espécies reativas é derivada do $O_2^{\bullet-}$, é de extrema importância que os papeis desempenhados por este radical sejam compreendidos no contexto fisiológico e das doenças.

1.3 Ânion Superóxido

A molécula de oxigênio (O_2) está presente em grandes quantidades no meio ambiente e possibilita a vida principalmente de organismos eucariontes. O poder oxidante desta molécula potencializa a conversão de energia presente nas ligações covalentes das moléculas de carbono em moléculas energéticas universais de adenosina trifosfato (ATP). Este processo, conhecido como fosforilação oxidativa, permitiu o desenvolvimento e a complexidade dos seres pluricelulares (WALLACE, 2010). Contudo, a mesma molécula de O_2, que possibilita a vida complexa, também apresenta toxicidade à integridade das células, um paradoxo da vida aeróbica. Este fato ocorre pela mesma característica que faz do O_2 uma molécula fisiologicamente necessária: a sua grande capacidade oxidante. De maneira geral, a molécula

de O_2 precisa ser reduzida para desempenhar seus papéis fisiológicos e gera, dessa forma, intermediários altamente reativos que são capazes de alterar a estrutura e a função de biomoléculas importantes, como lipídios, enzimas, e o DNA. Durante a cadeia respiratória, por exemplo, a redução tetravalente do O_2 tem como produto final H_2O. Porém, apesar de ser um processo relativamente seguro, esta redução ocorre de forma sequencial e gera intermediários altamente reativos, e que podem ser classificados como radicais livres ou como não radicais. O primeiro grupo é caracterizado pela presença de um elétron não emparelhado e inclui o ânion superóxido ($O_2^{\bullet-}$), o radical hidroxila ($HO^{\bullet}$), e o radical peroxil ($ROO^{\bullet}$). O segundo grupo é caracterizado, consequentemente, pela ausência de elétrons não emparelhados, mas também são derivados reativos do oxigênio, como o peróxido de hidrogênio (H_2O_2), o ácido hipocloroso (HOCl), e o peroxinitrito ($ONOO^-$) (BUETLER; KRAUSKOPF; RUEGG, 2004).

Como já citado, o principal mecanismo de geração dos intermediários reativos do oxigênio e das espécies reativas de oxigênio tem inicio com a redução do O_2 por um único elétron, gerando assim o radical $O_2^{\bullet-}$. Apesar de ser a etapa inicial na produção das demais espécies reativas e intermediários reativos do oxigênio, a participação do $O_2^{\bullet-}$ na sinalização celular e a sua contribuição na fisiopatologia das doenças inflamatórias têm sido pouco estudadas quando comparado ao papel bem estabelecido do H_2O_2 nestas e outras situações. Contudo, a literatura recente sugere que, em alguns processos fisiopatológicos, o $O_2^{\bullet-}$ tem papel crucial nas respostas mal-adaptativas e não o H_2O_2 (WANG et al., 2004). Apesar de ser considerado um agente citotóxico, o H_2O_2 apresenta pouca reatividade com biomoléculas e, além disso, sua concentração é estreitamente modulada por enzimas como CAT e Gpx (HALLIWELL; GUTTERIDGE, 1992; HALLIWELL et al., 2000). De fato, os efeitos tóxicos do H_2O_2 são desempenhados principalmente pelo $HO^{\bullet}$, e a formação deste último está relacionada à sinalização induzida pelo $O_2^{\bullet-}$ (HALLIWELL; GUTTERIDGE, 1984). A seguir, serão descritas as principais fontes do $O_2^{\bullet-}$ nas células de mamíferos, assim como seus efeitos moleculares e celulares, enfatizando sua relação com a inflamação e com a nocicepção.

1.3.1 Fontes de Ânion Superóxido

Diversas enzimas são responsáveis pela produção de $O_2^{\bullet-}$ nas células de mamíferos. Essa diversidade enzimática indica a importância crucial do $O_2^{\bullet-}$ aos processos fisiológicos, e

também a necessidade de uma produção local em razão da grande instabilidade que este ânion apresenta. Em condições basais, todas as células produzem $O_2^{\bullet-}$ principalmente na cadeia respiratória mitocondrial, especificamente no transporte de elétrons entre os complexos I e III (BOVERIS; CHANCE, 1973; RAHA; ROBINSON, 2000). Essa produção ocorre em taxas suficientemente baixas para seus efeitos deletérios que sejam controlados pelas únicas enzimas responsáveis por esta proteção, as superóxido dismutase (SOD). Através da atividade das SOD, há redução do $O_2^{\bullet-}$ em H_2O_2, uma espécie reativa não radical capaz de estimular a via do NF-κB. Apesar de não apresentar grande reatividade às biomoléculas, o $O_2^{\bullet-}$ pode sequestrar átomos de H de catecolaminas, DNA, RNA, ácidos graxos, esteroides, etc. Além disso, pode liberar o Fe(II) dos grupos Fe-S presentes nas metaloproteínas. O aumento na concentração de Fe(II) livre na célula favorece, por sua vez, que o produto da dismutação do $O_2^{\bullet-}$, o H_2O_2, escape da ação de enzimas antioxidantes como a CAT e forme o radical altamente reativo $HO^{\bullet}$ através da reação de Fenton (KEYER; IMLAY, 1996). A grande produção de H_2O_2 também induz o aumento de Fe(II) livre proveniente dos grupamentos heme e favorece a formação $HO^{\bullet}$ (HALLIWELL; GUTTERIDGE, 1986). O $HO^{\bullet}$, por sua vez, induz a peroxidação lipídica com consequente oxidação de GSH e ativação do NF-κB (CHAPPLE, 1997; ROVIN et al., 1997). Dessa forma, o $O_2^{\bullet-}$ tem sido associado à doenças inflamatórias, lesões por isquemia/perfusão, câncer, dor crônica e envelhecimento (FLINT; TUMINELLO; EMPTAGE, 1993). Esta importância do $O_2^{\bullet-}$ na fisiopatologia das doenças inflamatórias tem sido demonstrada pela eficácia dos tratamentos com miméticos da SOD (WANG et al., 2004). De fato, nosso grupo demonstrou que o tratamento com Tempol (mimético da SOD) reduz a dor e a inflamação induzidas por KO_2, um doador de $O_2^{\bullet-}$ (YAMACITA-BORIN et al., 2013). Diversas moléculas podem gerar $O_2^{\bullet-}$ enzimaticamente, como as xantina oxidases, ou através de auto-oxidação, como gliceraldeídos, $FADH_2$, hormônios e neurotransmissores. Porém, durante a inflamação, as NOX representam a principal fonte de $O_2^{\bullet-}$ (NATHAN, 2003).

1.3.2. Superóxido, Inflamação e Dor

Durante o processo inflamatório agudo, leucócitos são gradativamente ativados durante o recrutamento até o local do estímulo, onde produzem grandes quantidades de $O_2^{\bullet-}$. Como descrito anteriormente, essa produção ocorre principalmente através do complexo

enzimático das NOX2 localizado na membrana plasmática de fagócitos (neutrófilos, macrófagos, e células dendríticas) (HENDERSON; CHAPPEL, 1996). Nesse caso, a produção de $O_2^{\bullet-}$ desempenha papel microbicida essencial à eficácia contra os patógenos. Sua importância é observada nos quadros de infecção recorrente e não resolvida apresentados por pacientes com doença granulomatosa crônica. Estas enzimas utilizam NADPH para reduzir O_2 e gerar $O_2^{\bullet-}$ em grandes quantidades durante um processo (sugestivamente) chamado explosão respiratória. A localização destas enzimas permite que a produção de $O_2^{\bullet-}$ ocorra de forma localizada dentro de fagolisossomos, mas também de maneira dispersa no meio extracelular.

A produção de $O_2^{\bullet-}$ também tem papel crucial na ativação endotelial que pode ser dividido em duas fases. A fase inicial ocorre de maneira independente do NF-κB e está relacionada à produção de $O_2^{\bullet-}$ pelas células endoteliais e também pelos macrófagos. A sinalização via TLR/IRAK-4 nessas células (assim como em neutrófilos) leva à ativação rápida de NOX por meio da GTPase Rac1, e pela fosforilação direta da subunidade gp91phox por IRAK-4 (MAITRA et al., 2009; PACQUELET et al., 2007). O aumento na produção de $O_2^{\bullet-}$ induz a ativação das células endoteliais que passam a exibir um fenótipo pró-inflamatório, com aumento na expressão das moléculas de adesão. Além disso, tendo em vista a grande capacidade do $O_2^{\bullet-}$ em interagir com o NO, o controle da permeabilidade vascular depende do equilíbrio na produção desses dois mediadores, e o aumento na permeabilidade vascular está diretamente relacionado à produção excessiva de $O_2^{\bullet-}$. De fato, a inibição de NOS leva ao aumento do estresse oxidativo e também da permeabilidade vascular, e este efeito é revertido por inibidores da NOX ou antioxidantes (CHEN et al., 2011; SHARMA et al., 2009).

A fase tardia da resposta à produção de $O_2^{\bullet-}$ está relacionada à potencialização de vias de sinalização que levam à síntese de citocinas inflamatórias, como a via do NF-κB (KVIETYS; GRANGER, 2012). Com o recrutamento de neutrófilos, a produção de $O_2^{\bullet-}$ cresce rapidamente e sobrepõe as defesas antioxidantes endógenas, levando à depleção dos níveis de GSH e favorecendo dessa forma a persistência do processo inflamatório e a dor. Além disso, a ativação do NF-κB favorece também a síntese de mais $O_2^{\bullet-}$ e representa dessa forma um mecanismo de retroalimentação do processo inflamatório.

Alguns mediadores inflamatórios são também denominados mediadores hiperalgésicos intermediários ou finais. Os mediadores hiperalgésicos intermediários, como o TNF-α e a IL-

1β, não atuam diretamente nas fibras nociceptivas, mas induzem o aumento na produção de mediadores hiperalgésicos finais, como PGE_2, NGF e 5-HT (Figura 3). Os mediadores finais, por sua vez, atuam diretamente em receptores do neurônio nociceptivo e reduzem o limiar necessário à sua ativação, levando à hiperalgesia (VERRI JR. et al., 2006). A exposição prolongada a esses mediadores induz alterações na plasticidade neuronal que levam ao quadro de sensibilização de longa duração, com aumento na expressão de receptores e canais iônicos (EIJKELKAMP et al., 2010; FERRARI et al., 2012; MARCHAND; PERRETTI; MCMAHON, 2005). Aumentos na atividade e na expressão dos receptores TRPV1, por exemplo, são induzidos durante a inflamação periférica pela ação dos mediadores hiperalgésicos. O aumento da expressão, e a fosforilação desses receptores pelas proteínas quinase C (PKC) representa um importante mecanismo que leva à sensibilização periférica e central das fibras nociceptivas. A produção de $O_2^{\bullet-}$, assim como os mediadores hiperalgésicos, é capaz de modular a expressão, a fosforilação e a atividade de TRPV1, e, dessa forma, pode induzir a sensibilização dos nociceptores (SCHULTZ; USTINOVA, 1998; USTINOVA; SCHULTZ, 1994). Sendo assim, a produção de $O_2^{\bullet-}$ está relacionada não só com a inflamação persistente, mas também com o desenvolvimento da dor inflamatória. Porém, o papel do $O_2^{\bullet-}$ como mediador hiperalgésico ainda está pouco elucidado.

Considerando as informações apresentadas anteriormente, antioxidantes capazes de interferir nas diversas etapas dos efeitos induzidos pelo $O_2^{\bullet-}$, como a produção de $HO^{\bullet}$, o aumento Fe(II) livre, redução de GSH, e a ativação do NF-κB, representam importantes agentes com potencial terapêutico para doenças inflamatórias e para dor.

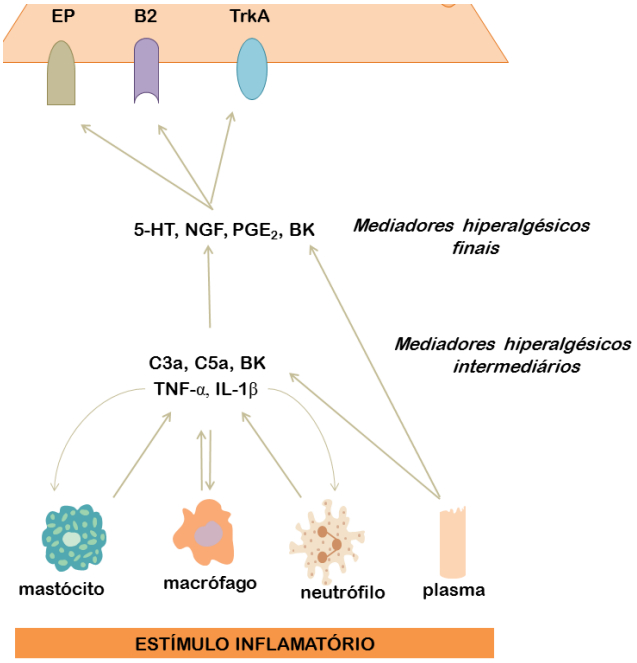

Figura 3 – Sensibilização periférica no local da inflamação. Mediadores hiperalgésicos intermediários que são produzidos na resposta inflamatória induzem o aumento na produção de mediadores hiperalgésicos finais. Os mediadores finais atuam diretamente em receptores do neurônio nociceptivo e reduzem o limiar necessário à sua ativação, levando à hiperalgesia.

1.4 PIRROLIDINA DITIOCARBAMATO

A pirrolidina ditiocarbamato (PDTC, CID: 65351) é um composto de baixo peso molecular (147,26) amplamente utilizado na pesquisa pré-clínica e na pesquisa clínica. A atividade biológica do PDTC foi inicialmente atribuída à inibição do estresse oxidativo e da ativação do NF-κB (BOYLE JR. et al., 1998; LIU; YE; MALIK, 1999). Apesar de ser amplamente utilizado como inibidor específico do NF-κB, a literatura tem demonstrado que o PDTC apresenta múltiplos alvos biológicos e, consequentemente, diversos mecanismos de ação. De maneira semelhante a outros ditiocarbamatos, o PDTC e seus derivados apresentam atividade quelante de íons metálicos, o que sugere sua potencial aplicabilidade na terapêutica de intoxicações por metais pesados, infecções (fúngica, bacteriana, e viral), doenças neurodegenerativas, e doenças neuroinflamatórias (SI et al., 2005; ZHANG; JIANG; ZUO, 2013). Durante a resposta inflamatória, o H_2O_2, produzido em grande quantidade após a dismutação do $O_2^{\bullet-}$ pelas SOD, induz a degradação do IκBα e, consequentemente, a liberação do heterodímero p65/p50 do NF-κB (GLOIRE; PIETTE, 2009). Vale lembrar que os efeitos

deletérios do H_2O_2 ocorrem na sua maioria pelo seu produto $HO^{\bullet}$. A produção de $O_2^{\bullet-}$, juntamente com o aumento de H_2O_2, induz a produção de $HO^{\bullet}$ espontaneamente (na presença de luz ultravioleta) ou através da interação com íons metálicos, principalmente Fe(II) (reação de Fenton), e que o $HO^{\bullet}$ leva à peroxidação lipídica com consequente oxidação de GSH e, por fim, sinaliza vias pró-inflamatórias importantes (HALLIWELL; GUTTERIDGE, 1990). Além disso, o H_2O_2 não favorece a formação de $HO^{\bullet}$ apenas sendo substrato na reação de Fenton, mas também aumenta a disponibilidade de Fe(II) que é liberado pela reação de H_2O_2 com grupamentos heme de mioglobina e hemoglobina (HALLIWELL; GUTTERIDGE, 1986). Dessa forma, o PDTC representa um composto útil na avaliação da participação do $O_2^{\bullet-}$ em estímulos inflamatórios e modelos animais de doenças humanas. O efeito antioxidante do PDTC ocorre pelo conjunto de 3 mecanismos principais. Primeiro, o PDTC é capaz de sequestrar diretamente ambos os radicais $O_2^{\bullet-}$ e $HO^{\bullet}$ (SHI et al., 2000). Segundo, como agente quelante, o PDTC reduz a disponibilidade de Fe(II) livre e, dessa forma, impede a conversão de H_2O_2 em radicais extremamente reativos como o $HO^{\bullet}$. Terceiro, o PDTC induz a oxidação do NF-κB no núcleo, mais especificamente na subunidade p50 (Cys-62), o que impede sua ligação ao DNA e representa um mecanismo de inibição da atividade do heterodímero p50-p65 após sua translocação para o núcleo (BRENNAN; O'NEILL, 1996).

2 OBJETIVOS

2.1 OBJETIVO GERAL

Avaliar a participação do NF-κB na inflamação e na hiperalgesia induzida pelo superóxido de potássio.

2.2 OBJETIVOS ESPECÍFICOS

- Avaliar o efeito anti-inflamatório e anti-nociceptivo do tratamento com PDTC em modelos de dor manifesta e hiperalgesia induzida pelo superóxido de potássio;

- Avaliar a presença de neutrófilos e macrófagos no processo inflamatório e hiperalgésico induzido pelo superóxido de potássio;

- Verificar o perfil de citocinas inflamatórias e hiperalgésicas, e os níveis de glutationa reduzida no local do estímulo com superóxido de potássio e na medula espinal dos grupos tratados com PDTC ou com veículo;

- Demonstrar a participação da via do NF-κB no local do estímulo periférico e sua relação com as demais respostas inflamatórias, locais e sistêmicas.

3 ARTIGO PARA PUBLICAÇÃO

Este trabalho foi realizado no Laboratório de Dor, Inflamação, Neuropatia e Câncer, da Universidade Estadual de Londrina. Os resultados estão descritos no artigo “Superoxide anion-induced inflammation and pain: the role of NF-κB”.

As formatações do artigo seguem as normas da revista Free Radical Biology & Medicine (Anexo).

Pyrrolidine dithiocarbamate inhibits inflammation and pain induced by potassium superoxide, a superoxide anion donor: role of cytokines, oxidative stress, and NF-κB

Felipe A. Pinho-Ribeiro, Victor Fattori, Ana C. Zarpelon, Sergio Marques Borghi, Thacyana Teixeira de Carvalho, Thiago M. Cunha, and Waldiceu A. Verri, Jr.

Highlights

-Intraplantar (i.pl.) injection of potassium superoxide (KO_2) induces inflammation and pain.

-NF-κB inhibitor PDTC prevents inflammation, hyperalgesia, pain, and oxidative stress induced by KO_2

-KO_2 intraplantar injection induces local IκBα degradation

-NF-κB is required in KO_2-induced paw edema and leukocyte recruitment

Pyrrolidine dithiocarbamate inhibits inflammation and pain induced by potassium superoxide, a superoxide anion donor: role of cytokines, oxidative stress, and NF-κB

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ABSTRACT

Superoxide anion radicals are produced in large rates during inflammation and thus sensitize nociceptive fibers leading to the pathological pain states of hyperalgesia or allodynia. In the present study, the peripheral and spinal roles of NF-κB in superoxide-induced inflammation, pain, hyperalgesia, and oxidative stress, were investigated using the superoxide anion-donor potassium superoxide (KO_2) as stimulus in mice. The subcutaneous pre-treatment with pyrrolidine dithiocarbamate (PDTC, 3 – 100 mg/kg, 100 µL, 1 h), a widely used NF-κB inhibitor, reduced in a dose-dependent manner the superoxide anion-induced hyperalgesia to mechanical and thermal stimuli, the edema formation, and also the recruitment of neutrophils and macrophages in the site of stimulus. Moreover, the overt pain-like behavior (writhings, paw flinching, and paw licking) induced by superoxide anion was reduced by PDTC treatment. The PDTC treatment reduced total leukocyte, monocyte and neutrophil recruitment to the peritoneal cavity, which was consistent with the reduction of superoxide-induced glutathione (GSH) depletion and cytokine (IL-1β, TNF-α, and IL-10) production in plantar skin tissue. At the spinal level, (L4 – L5), PDTC also inhibited the superoxide-modulation of cytokine (IL-1β, TNF-α, and IL-10) and GSH levels. The superoxide-induced degradation of NF-κB inhibitor alpha (IκBα) levels in the plantar skin were inhibited by PDTC treatment at 1 and 3 h after superoxide stimulus. Thus, the present study reveals that superoxide anion-induced inflammation and pain depend on oxidative stress, cytokine production and NF-κB activation.

Key words: Potassium superoxide, pyrrolidine dithiocarbamate, inflammation, pain, NF-κB

Introduction

Many physiological processes that use molecular oxygen, such as oxidative phosphorylation of mitochondrial respiratory chain, depend on the high reactivity of its derivatives, namely reactive oxygen intermediates (ROI). These one-electron reduction products of oxygen include, successively, superoxide anion radical ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($HO^{\bullet}$) [1]. Under physiological conditions, superoxide production occurs at low rates in cells, but is upregulated by pro-inflammatory signaling molecules such as cytokines (TNF-α and IL-1β), and the damage- and pathogen-associated molecular patterns (e.g. hyaluronan fragments, endotoxin). The inflammation-induced increase of superoxide levels plays important roles in cellular signaling and host defense against infections, but also provoke tissue damage. In fact, mammal cells produce several intracellular and extracellular specific molecules and enzyme systems that are responsible for sensing and/or inactivating superoxide anion to prevent detrimental impact of superoxide [2]. The major antioxidant mechanism that interferes directly with superoxide formation is the superoxide dismutase (SOD) enzymes that catalyze the dismutation of superoxide to non-radical hydrogen peroxide.

During the inflammatory process, recruited neutrophils and macrophages produce large amounts of superoxide anion through activation of (phagocytic) NADPH oxidase 2 (NOX 2) complex, an essential tool against infections that is dependent on NF-κB activation [3]. The importance of this intentional superoxide production, known as respiratory burst, is evidenced by recurrent and unresolved infections that occur in patients with chronic granulomatous disease [4]. However, the excessive superoxide production in an inflammatory milieu can overwhelm the endogenous anti-oxidant defenses. Such an imbalance leads to oxidation of cysteine groups and trigger maladaptive signaling that increases edema formation [5],

neutrophil recruitment, cytokine production, and pain [6, 7]. Sulfenic acid, the early product of cysteine oxidation, reacts with glutathione (GSH) tripeptide to form an S-glutathionated protein, an essential antioxidant mechanism that protects biomolecules against irreversible oxidation and that is also implicated in cell signaling [8]. Thus, GSH depletion is a marker of oxidative stress-related conditions as occur during exacerbated inflammation [9]. Despite the widely studied roles of hydrogen peroxide in cellular signaling, the emerging literature focusing on SOD activity suggests that superoxide anion is a critical mediator implicated in nociception and pathological pain [10]. However, the underlying mechanisms by which superoxide induces pain and hyperalgesia are still not fully understood. In order to investigate the pro-inflammatory and pro-nociceptive effects of superoxide anion, our group has standardized the nociceptive stimuli by injection of potassium superoxide (KO_2) solution [11], a superoxide anion-donor compound [12]. In this model, potassium superoxide injection induces inflammation, hyperalgesia, overt pain-like behavior, and oxidative stress [11].

Taking into account the above mentioned evidence, the present study investigated the role of NF-κB signaling pathway, a key redox-sensitive component of inflammation and pain, in potassium superoxide-induced pain and inflammation. The results presented in this study reveal for the first time that NF-κB activation is required in superoxide-induced inflammation, hyperalgesia, and overt pain-like behavior by increasing both local and spinal oxidative stress and cytokine production.

Materials and Methods

Animals

The experiments were performed on male Swiss mice (20-25 g, Londrina State University, Londrina, PR, Brazil) housed in standard plastic cages (six mice 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- and light-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 Londrina State University (process 71.2012.68).

Drugs and reagents

Potassium superoxide (KO_2) 96.5% was purchased from Alfa Aesar (Ward Hill, MA, USA). Phosphate buffered saline (PBS), *ortho*-dianisidine dihydrochloride, 4-nitrophenyl-N-acetyl-β-D-glucosaminide, pyrrolidine dithiocarbamate (PDTC), trichloroacetic acid (TCA), hexadecyltrimethylammonium bromide (HTAB), glutathione (GSH), EDTA sodium salt, ferric chloride hexahydrate, and 2,4,6-tripiridil-s-triazine (TPTZ) were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Experimental protocols

Hyperalgesia to mechanical and thermal stimuli, and paw edema formation were induced by KO_2 (30 µg, 25 µL, i.pl.) injection, and evaluated at 0.5 – 7 h after KO_2 or sterile saline (25 µL, i.pl.) injection. The animals were euthanized immediately after the last measurement (7 h), and tissue samples from the plantar skin were collected and used in myeloperoxidase (MPO) and N-acetyl-β-D-glucosaminidase (NAG) activity assays. The number of paw flinches and the time spent licking the paw were measured immediately after a single KO_2 (30 µg, 25 µL, i.pl.) or sterile saline (25 µL, i.pl.) injection for 30 min. The number of writhings

was measured immediately after KO_2 (1 mg, 150 µL, i.p.) or saline (150 µL, i.p.) injection. GSH and cytokine (IL-1β, TNF-α, and IL-10) levels were determined in tissue samples from plantar skin and spinal cord (L4 – L5) 3 h after KO_2 (30 µg, 25 µL, i.pl.) or sterile saline (25 µL, i.pl.) injection. IκBα levels were determined 0.5 – 3 h after KO_2 (30 µg, 25 µL, i.pl.) or sterile saline (25 µL, i.pl.) injection by western blotting. In another approach, peritoneal cells were harvested in 1.5 mL of PBS, 3 h after KO_2 (30 µg, 25 µL, i.p.) or sterile saline (25 µL, i.p.) injection, and the samples were used in total and differential cell counts. The doses of KO_2 used in each test were selected based on a previous work of standardization [11]. PDTC (3 – 100 mg/kg, 100 µL, s.c.) or vehicle (sterile saline, 100 µL, s.c.) treatment was given 1 h before KO_2 or saline injection. This protocol was used in all experiments.

Electronic pressure meter test of mechanical hyperalgesia

The test consisted of evoking a hind paw reflex with a hand-held force transducer (electronic von Frey anesthesiometer; Insight, Ribeirão Preto, SP, Brazil) adapted with a 0.5 mm^2 polypropylene tip. Detailed methodology was previously described by our group [11]. The results are expressed by delta (Δ) of withdrawal threshold (in g) calculated by subtracting the basal mean measurements from the mean measurements at the indicated time points after stimulus.

Hot Plate Test

The test was performed as reported previously [11]. Briefly, mice were placed the hot plate apparatus (EFF 361, Insight Equipamentos, Ribeirao Preto, SP, Brazil) maintained at 55 °C. The reaction time was scored when the pain reactions of hind paw licking or flinching occurred in the injected paw. As the basal latencies were always smaller than 20 s (between 15 and 20 s), a maximum latency (cut-off) was set at 20 s to avoid tissue damage.

Paw edema

The paw edema formation was measured using a caliper (Digmatic Caliper, Mitutoyo Corporation, Kanagawa, Japan). Values of paw thickness are expressed as the difference (Δ mm) between the values obtained just before (basal) and after stimulus injection.

MPO activity

The neutrophil recruitment to the paw skin was evaluated by the MPO assay [13]. Mice were euthanized, and the plantar skin samples were collected and homogenized by a tissue tearor (Bio Spec Products, Bartlesville, OK) in 400 µl of ice-cold K_2HPO_4 buffer (50 mM, pH 6.0) containing 0.5% of HTAB. After that, homogenates were centrifuged (16 100 *g*, 2 min, 4 °C) and aliquots of 30 µL of supernatant were placed in a 96-well plate and mixed with 200 µL of ice-cold K_2HPO_4 buffer (50 mM, pH 6.0) containing 0.0167% *ortho*-dianisidine dihydrochloride and 0.05% H_2O_2. After 5 min of incubation, the absorbance values were measured at 450 nm (Multiskan GO Microplate Spectrophotometer, ThermoScientific, Vantaa, Finland). The MPO activity of samples was obtained by comparing the absorbance with a standard curve of neutrophils, and the results were presented as MPO activity (number of neutrophils × 10^3/mg of tissue).

NAG activity

NAG (N-acetyl-β-D-glucosaminidase) activity was determined by an adapted colorimetric method previously described [14]. Briefly, aliquots of 20 µL of supernatant obtained previously in MPO assay were placed in a 96-well plate, followed by the addition of 80 µL of PBS (50 mM, pH 6.0). The reaction was initiated by the addition of 100 µL of 4-nitrophenyl N-acetyl-β-D-glucosaminide (NAG substrate, 2.24 mM). The plate was incubated at 37 °C for 10 min, and the reaction was stopped by addition of 100 µL of glycine buffer (0.2 M, pH

10.6). The absorbance was determined spectrophotometrically at 400 nm (Multiskan GO Microplate Spectrophotometer, ThermoScientific, Vantaa, Finland). The NAG activity of samples was obtained by comparing the absorbance with a standard curve of macrophages, and the results were presented as NAG activity (number of macrophages × 10^3/mg of tissue).

Writhing Response Tests

Mice were placed individually in large glass cylinders immediately after stimulus injection, and the total number of writhings was counted for 20 min [11]. The pain intensity was expressed as a cumulative number of writhings.

Paw flinches and licking test

The number of paw flinches, and the time spent licking the stimulated paw were both determined immediately after stimulus injection for 30 min [11]. Results were expressed as the cumulative number of paw flinches, or as the total time spent licking the paw.

Leukocyte recruitment in the peritoneal cavity

Mice were euthanized 3 h after KO_2 (30 µg, 25 µL, i.p.) or saline (25 µL, i.p.) injection, and the peritoneal cells were harvested with 1.5 mL of PBS [11]. Aliquots of 20 µL were mixed with 180 µL of Turk's solution, and the total cell counts were performed in Neubauer chamber. Differential cell counts were performed on microscope slides using the Panoptic staining kit for histological analysis (Laborclin, Pinhais, PR, Brazil). The values were expressed as the total number of cells ×10^6 per cavity. Total and differential cell counts were both performed under a light microscope (400x magnification, Olympus Optical Co., Hamburg, Germany).

GSH assay

Mice were euthanized and the tissue samples from plantar skin and spinal cord were collected 3 h after KO_2 (30 µg, 25 µL, i.pl.) or saline (25 µL, i.pl.) injection. Tissue samples (pool of 2) were homogenized in ice-cold EDTA (2 mM) buffer using a tissue tearor (Bio Spec Products, Bartlesville, OK, USA). The GSH colorimetric assay was performed as previously described [15], and the absorbance values were compared with a standard curve of GSH.

IL-1β, TNF-α, and IL-10 levels

The cytokine levels were measured in tissue samples from plantar skin and spinal cord. The samples were collected and homogenized in 500 µL of ice-cold PBS containing protease inhibitors. The IL-1β, TNF-α, and IL-10 levels were determined as described previously [16] by enzyme-linked immunosorbent assay (ELISA) Ready-SET-Go! kits (eBioscience, San Diego, CA), and the results were expressed as pg of cytokine per mg of tissue.

Western blotting

Equal amounts of protein (20 µg) from the plantar skin tissue were separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and transferred to a nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA, USA). A molecular weight standard (Bio-Rad Laboratories) was run in parallel to estimate molecular weight. Membranes were blocked overnight at 4 ºC in Tris-buffered saline-Tween (20 mM Tris-HCl, pH 7.5, 500 mM NaCl, 0.1% Tween 20; TBST) containing 5% of non-fat dried milk. After blocking, the membranes were incubated at 4 °C overnight, with anti-IκBα (1:1000), or anti-β-actin internal control (1:1000) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), diluted in TBST containing 5% of non-fat dried milk. The membranes were then incubated with appropriate secondary antibody conjugated with peroxidase (1:5000) diluted in TBST containing 5% of non-fat dried milk, and incubated at room temperature for 60 minutes. Finally, the bands were visualized using a chemiluminescence-based ECL system (Amersham Biosciences,

Piscataway, NJ, USA) and exposed to an X-ray film for 30 minutes (Eastman Kodak, Rochester, NY, USA). A computer-based imaging system (Image J, National Institute of Health, Bethesda, MD, USA) was used to measure the optical density of the bands.

Statistical Analysis

Results are presented as means ± SEM of measurements made on 6 mice in each group. Two-way analysis of variance (ANOVA) was used to compare the groups and doses at all times (curves). 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 Tukey's t-test was performed for each time. On the other hand, when the nociceptive responses were presented as total values at the indicated time period, the differences between responses were evaluated by one-way ANOVA followed by Tukey's t-test. Statistical differences were considered to be significant at $P < 0.05$.

Results and discussion

PDTC inhibits potassium superoxide (KO_2)-induced hyperalgesia and inflammation.

In a previous work [11], it has been shown that KO_2 (superoxide anion donor) injection induces hyperalgesia to mechanical and thermal stimuli, edema formation, and leukocyte recruitment in a dose-dependent manner. In order to investigate the role of NF-κB, a redox-sensitive transcription factor, in the pro-inflammatory and pro-hyperalgesic effects of superoxide anion, mice were pre-treated with different doses of PDTC (3 – 100 mg/kg, 100 µL, s.c.), a widely used NF-κB inhibitor, 1 h before KO_2 injection (Figure 1). As expected, KO_2 injection (30 µg, 25 µL, i.pl.) reduced the nociceptive thresholds to mechanical (Figure 1A) and thermal (Figure 1B) stimuli, and induced paw edema formation (Figure 1C) at all evaluated time points. Moreover, the recruitment of neutrophils (Figure 1D) and macrophages (Figure 1E) was also induced by KO_2 injection. Corroborating the initial hypothesis of this study, PDTC administration inhibited the KO_2-induced hyperalgesia (Figures 1A and B), edema formation (Figure 1C), and neutrophil (Figure 1D) and macrophage (Figure 1E) recruitment in a dose-dependent manner. The most effective dose was 100 mg/kg of PDTC. Therefore, the dose of 100 mg/kg was selected for the next experiments. Taken together, these data suggest that superoxide-induced inflammation and inflammatory pain are dependent on NF-κB activation, a signaling pathway that is shared by several sterile and non-sterile stimuli [17], and may represent a possible mechanism that, for instance, leads to the exacerbation and perpetuation of pain during infection and auto-immunity. During acute inflammatory response, endothelial cells, neutrophils, and macrophages produce and release large amounts of superoxide anion that, in turn, facilitates leukocyte transmigration by several mechanisms such as activation of matrix metalloproteinases (MMP) and oxidant-mediated fragmentation of hyaluronan [18, 19]. The superoxide-induced production of low-molecular mass hyaluronan (a damage-associated molecular pattern), for example, can induce NF-κB

activation and, consequently, represents one possible mechanism by which KO_2-released superoxide anion triggers inflammation [20]. In brief, the results are suggestive that the production of NF-κB-related pro-inflammatory and pro-hyperalgesic molecules (such as TNF-α, IL-1β, and ROI) could lead to the inflammatory signals observed in this model (Figure 1).

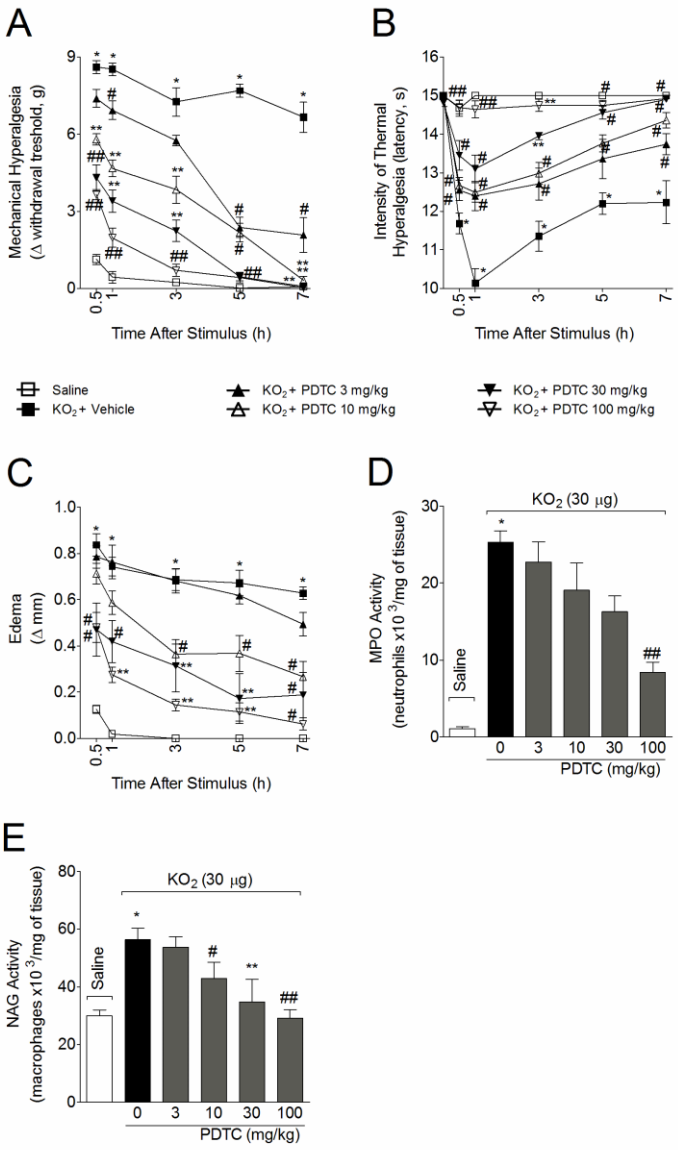

Figure 1

KO_2-induced overt pain-like behavior depends on NF-κB activation.

The role of NF-κB in the superoxide-induced overt pain-like behavior of writhings, paw flinches, and paw licking was also evaluated. Treatment with PDTC (100 mg/kg, 100 µL, s.c.) showed significant reduction of KO_2-induced nociceptive behaviors of writhings (Figure 2A), and paw flinching (Figure 2B) and licking (Figure 2C). Redox active molecules can directly modulate the activity of TRPV1 channels [21, 22]. Nevertheless, the present data suggest that there is also NF-κB-dependent activation of nociceptive fibers by superoxide anion.

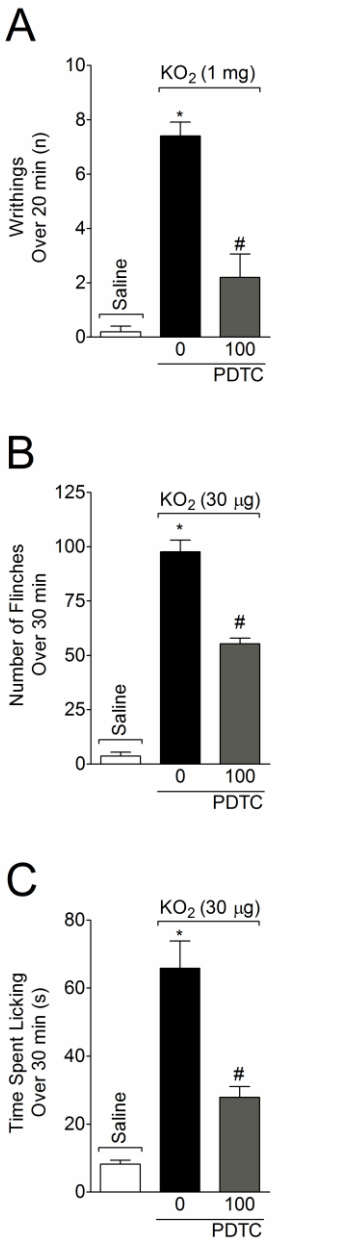

Figure 2

KO_2-induced leukocyte recruitment depends on NF-κB.

The leukocyte recruitment in the peritoneal cavities from PDTC-treated mice was measured and compared with samples from vehicle-treated mice (Figure 3). Mice were treated with PDTC (100 mg/kg, 100 µL, s.c.) 1 h before KO_2 (30 µg, 25 µL, i.p.) or saline (25 µL, i.p.) injection. Peritoneal cells were harvested in 1.5 ml of PBS 4 h after stimulus. Total cell counts (Figure 3A) were determined in Neubauer chamber, and differential cell counts (Figure 3B and C) were determined using Panoptic staining kit. Corroborating the results obtained in tissue samples from the plantar skin (MPO and NAG assays, Figure 1D and E), PDTC treatment reduced the superoxide-induced recruitment of both polymorphonuclear (Figure 3B) and mononuclear (Figure 3C) cells in the peritoneal cavity. As discussed before, these results are suggestive that superoxide can trigger the inflammatory response even when the pain-like behavior is not evident. Thus, indicating that pain and leukocyte recruitment are independent effects, but both depend on NF-κB activation.

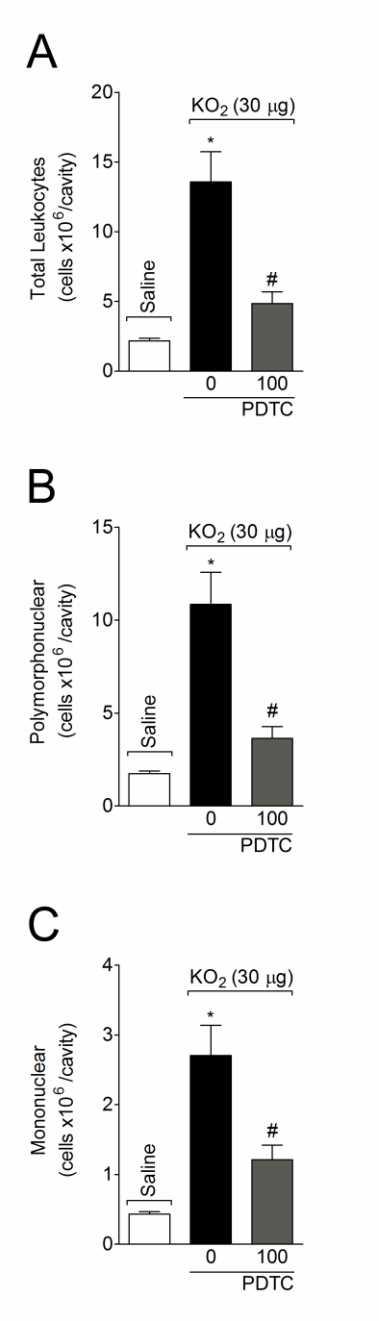

Figure 3

KO_2 induces local and spinal oxidative imbalance in a NF-κB-dependent manner.

The activation of NF-κB is regulated by tyrosine kinases and phosphatases that are, in turn, regulated by redox status. Once this pathway has been activated, the heterodimer p65/p50 induces the transcription of early pro-oxidant enzymes, which includes NOX subunit gp91phox, pro-inflammatory cytokines, and pro-hyperalgesic mediators. NOX enzymes are

the major responsible for the oxidative stress during inflammatory responses, a process that leads to central and peripheral sensitization [23]. GSH tripeptides are the most abundant cellular antioxidant molecules and considered indicative of redox status in biological samples. Thus, the levels of GSH were determined in plantar and spinal tissue samples from PDTC- or vehicle- treated mice, 3 h after KO_2 (30 µg, 25 µL, i.pl.) stimulus (Figure 4). The KO_2-induced depletion of GSH levels was inhibited by PDTC treatment in plantar tissue samples (Figure 4A), which is in line with its anti-inflammatory effects already presented. Curiously, the KO_2 stimulus induced an increase in GSH at the spinal level, which was reduced by PDTC treatment (Figure 4B). These results suggest that, peripherally, oxidative stress occurs not only by KO_2-derived superoxide anion, but also by neutrophil- and macrophage-derived ROI in a NF-κB-dependent manner. In this case, the excessive production of ROI by recruited leukocytes overwhelms the antioxidant defenses, and triggers nociceptive impulses.

On the other hand, the peripheral activation of first-order nociceptive fibers leads to the transmission of nociceptive impulses into the dorsal horn of spinal cord. Glial cells continually inspect neuronal activity and can be activated by neuronal signals that are released during pain transmission, including glutamate, substance P and fractalkine [24]. This glial activation leads to release of NF-κB-related cytokines (TNF-α, IL-1β and IL-10) and ROI [25, 26], but also induces protective mechanisms through activation of redox-responsive NF-E2-related factor 2 (Nrf2) signaling. In glial cells, the Nrf2 pathway upregulates the production of enzymes that are related to increased GSH synthesis such as xCT cystine antiporter, γ-glutamylcysteine synthetase (γ-GCS), GSH synthase, glutathione S-transferase and glutathione reductase). This process has been shown to be necessary and sufficient to protect neurons against oxidative damage and glutamate toxicity [27]. Despite the fact that glial cells also produce pro-inflammatory (TNF-α, IL-1β) and pro-oxidant mediators in response to nociceptive transmission inputs, the KO_2-induced oxidative stress in spinal cord seems to be

less intense when compared with the peripheral response where activated leukocytes are being continuously recruited. Thus, this KO_2-induced low-intensity spinal inflammation may allow to be reversed by glial antioxidant mechanisms. In response to TNF-α-induced GSH depletion, for example, there is fast γ-GCS activation. This enzyme is physiologically inactive due to binding to GSH, but the oxidative stress condition leads to its activation when the GSH-bound is released from the enzyme. Moreover, the γ-GCS mRNA is found upregulated under oxidative stress conditions, as discussed before. Thus, the TNF-α-induced enzymes of GSH cycle may explain the higher levels of GSH found in vehicle-treated mice when compared to non-stimulated and PDTC-treated mice (Figure 4B).

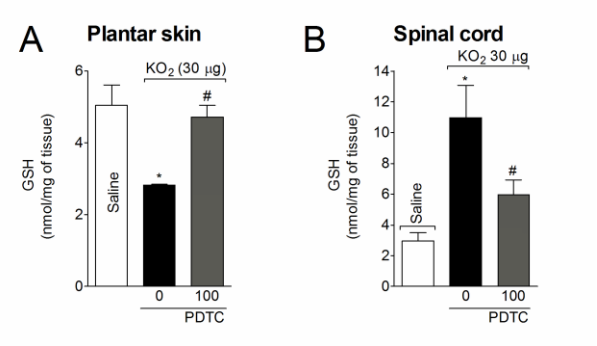

Figure 4

Superoxide-induced local and spinal cytokine production depends on NF-κB activation.

The pro-inflammatory cytokines TNF-α and IL-1β are also considered hyperalgesic mediators. Some of its activities include the induction of direct-acting hyperalgesic mediator PGE_2 [28]. These cytokines can also sensitize nociceptive fibers at both peripheral and spinal levels [25, 26, 29, 30]. Furthermore, the production of TNF-α and IL-1β, as well as IL-10, are upregulated during inflammation in a NF-κB-dependent manner and therefore, can be considered indicative of NF-κB activation. To further investigate the role of NF-κB in superoxide-induced cytokine production, the levels of IL-1β, TNF-α, and IL-10 were

determined in tissue samples from plantar skin and spinal cord of PDTC- and vehicle-treated mice, 3 h after KO_2 (30 µg, 25 µL, i.pl.) or saline (25 µL, i.pl.) injection (Figure 5). The KO_2-induced increase in local (Figure 5A, C and E) and spinal (Figure 5B, D, and F) levels of IL-1β, TNF-α and IL-10 was reduced significantly by PDTC treatment. The reduction of all measured cytokines suggests that superoxide-induced inflammation and inflammatory pain requires the activation of NF-κB at the local of stimulus. NF-κB is also activated at the spinal level where glial cells are the major sources of cytokines. Nevertheless, the peripheral activation of NF-κB and consequent cytokine production induce the activation at spinal levels, thus, the inhibition of superoxide-induced cytokine production at the spinal cord by PDTC treatment does not necessarily indicates a spinal effect, but rather may be a consequence of peripheral inhibition of increased spinal inputs.

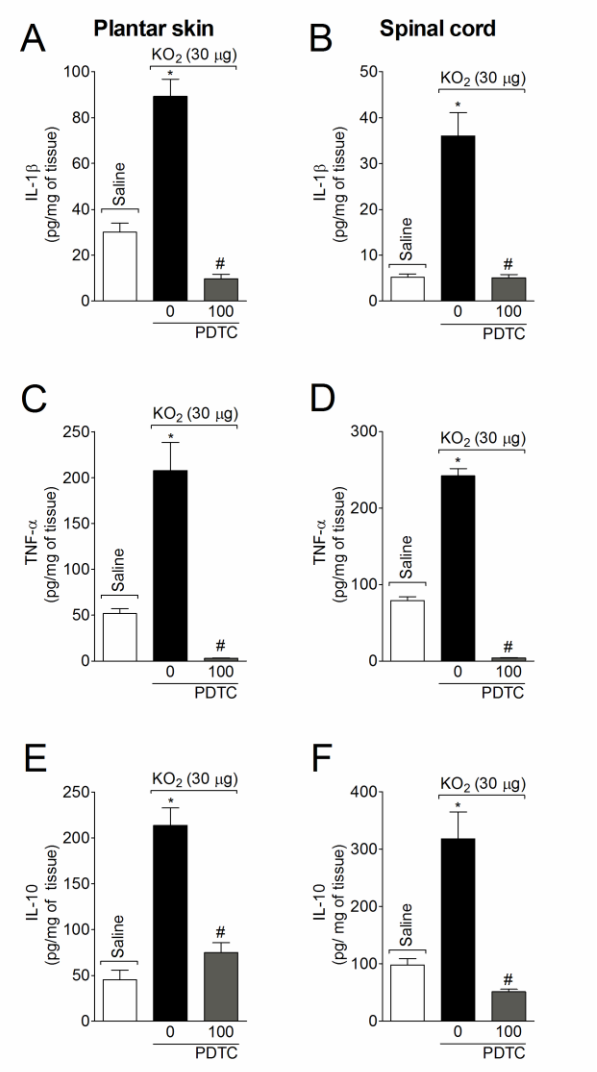

Figure 5

PDTC inhibits superoxide-induced local reduction of IκBα levels in a time-dependent manner.

Typically, the NF-κB heterodimers p65/p50 activity is found inhibited by IκBα proteins. These inhibitory molecules bind to NF-κB and mask their DNA binding domains. During the canonical activation NF-κB pathway, which occurs by several pro-inflammatory stimuli (e.g. TNF-α, ROI, endotoxin), the IκBα is phosphorylated by IκB kinase (IKK) complex, ubiquitinated, and then degraded by proteasome. This process unmasks the DNA binding activity of p65/p50 and lead to transcription of pro-inflammatory genes [17]. Thus, the activation of IKK complex was evaluated indirectly by measuring IκBα levels in different

time points (Figure 6). Mice were pre-treated with PDTC (100 mg/kg, 100 µL, s.c.) or vehicle (saline, 100 µL, s.c.) 60 min before KO_2 (30 µg, 25 µL, i.pl.) or saline (25 µL, i.pl.) injection, and tissue samples from the plantar skin were collected 0.5 – 3 h after stimulus. The superoxide-induced reduction of IκBα levels were inhibited by PDTC treatment at 1 and 3 h after stimulus, but no inhibition was observed at 0.5 h (Figure 6). In order to understand the anti-inflammatory and anti-hyperalgesic effects PDTC at 0.5 h, it is important to consider some NF-κB-independent pro-inflammatory signaling pathways induced by superoxide anion and its derivatives. SOD enzymes are constitutively present in cells and in the extracellular matrix, which suggests that part of KO_2-derived superoxide anion was converted immediately in hydrogen peroxide, a cell permeable molecule that can induce IκBα degradation and NF-κB activation [31]. The antioxidant activity of PDTC has been related to its iron-chelating properties, thus preventing the hydrogen peroxide reduction to the highly deleterious hydroxyl radical through the Fenton reaction [32]. PDTC also scavenges superoxide and hydroxyl radical [33]. Although hydrogen peroxide activates NF-κB, this effect is reversible and can be attenuated by antioxidant endogenous components, such as CAT and Gpx. In contrast, superoxide anion and hydroxyl radicals lead to irreversible and fast oxidation of biomolecules [34]. Some of these effects occur within 30 min (NF-κB independent) and include: enhanced vascular permeability, activation of matrix metalloproteases, endothelial mobilization of Weibel-Palade bodies and phospholipase A2-derived platelet-activating factor production, hyaluronan fragmentation, and transient receptor potential activation in nociceptive neurons [35, 36]. In this sense, PDTC may not prevent this partial production of hydrogen peroxide by SOD and the hydrogen peroxide-related NF-κB activation at 0.5 h, but could prevent the superoxide- and hydroxyl-related effects at early (NF-κB independent) and delayed (> 120 min, NF-κB dependent) phases of inflammation. Furthermore, the results are suggestive that, despite of its reported antioxidant activity, the protective effects of PDTC observed in this

model occur by mechanisms downstream of IκBα. In fact, PDTC exhibits a pro-oxidant effect (through its thiol group), which is suggested to be the mechanism of specific inhibition of NF-κB activity [37]. PDTC interfere with DNA binding activity of NF-κB through oxidation of a sensitive thiol group of p50 (Cys-62) subunit, without preventing the NF-κB nuclear translocation as DNA binding was restored in nuclear extracts after 2-mercaptoethanol addition. While pro-oxidant signals are required to induce NF-κB activation in the cytosol, the reducing condition in the nucleus is necessary to maintain p50 cysteine-62 on its reduced and active form [38]. This may explain why PDTC could not prevent the IκBα reduction that occurs at 0.5 h, when KO_2-derived superoxide anion is the major inflammatory stimulus rather than the NF-κB-induced transcription of pro-inflammatory mediators. Moreover, PDTC could be inhibiting other downstream mechanisms of NF-κB activation, such as p65 (Ser-276) phosphorylation [31]. This hypothesis is corroborated by the fact that PDTC-treated groups showed significant inhibition of IκBα degradation since 1 h, suggesting that NF-κB-induced production of pro-inflammatory mediators were inhibited at 0.5 h.

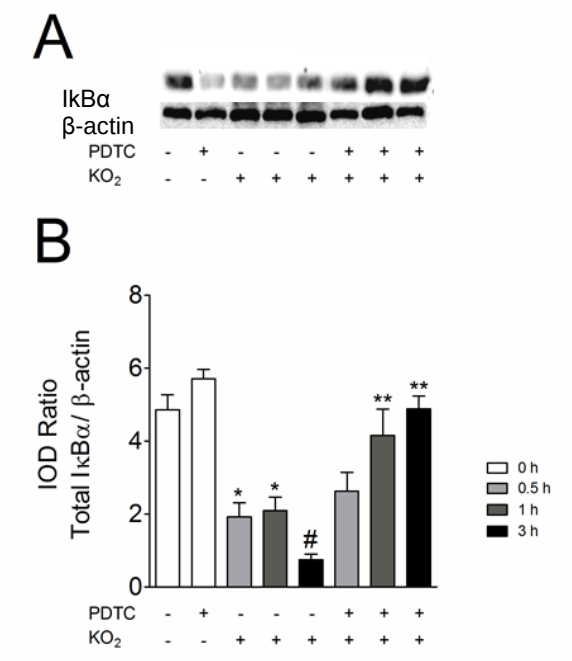

Figure 6

Conclusion

The peripheral stimuli with KO_2, a superoxide anion-donor, induced acute overt pain-like behaviors (between 0 and 30 min after injection), and produced the inflammatory signs of hyperalgesia (thermal and mechanical), edema formation, and neutrophil and macrophage recruitment (between 0.5 and 7 h before injection). Its mechanisms include the early local IκBα degradation (0.5 – 3 h after injection), GSH depletion, and production of the NF-κB-related cytokines TNF-α, IL-1β, and IL-10 at the peak of hyperalgesia (3 h after injection) at both peripheral and spinal levels. Moreover, the results showed that GSH levels are upregulated in spinal tissue after peripheral stimulus with KO_2, where GSH levels were found depleted. Finally, PDTC exhibited protective effects by mechanisms downstream of IκBα degradation that should be further investigated. Thus, the study contributes to understanding the role of superoxide anion radicals in peripheral and spinal sensitization of nociceptive fibers during the inflammatory process, and the underlying mechanisms of pathological pain development.

Declaration of Interest Section

The authors declare no conflict of interest.

Author contributions

FAPR, VF, ACZ, SMB and TTC performed experiments; FAPR, WAVJ analyzed and interpreted data; TMC provide analytical tools; RC and WAVJ design and conception of study; FAPR and WAVJ wrote the paper; FAPR, VF, SMB, ACZ, TTC, TMC and WAVJ approved the final version to be published.

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Figure Captions

Fig. 1. KO_2 stimulus depends on NF-κB activation to induce hyperalgesia and inflammation. Mice were treated with PDTC (3 – 100 mg/kg, 100 µL, s.c.) or vehicle (saline, 100 µL, s.c.) 60 min before the potassium superoxide (KO_2) stimulus. The nociceptive thresholds to mechanical (A) and thermal (B) stimuli, and the paw edema formation (C) were measured 0.5 – 7 h after KO_2 injection (30 µg, 20 µL, i.pl.). Neutrophil (D) and macrophage (E) recruitment in the injected paw was assessed 7 h after stimulus. Results are expressed as means ± SEM, n = 6 mice per group [*p < 0.05 vs. saline control; $^{\#}p$ < 0.05 vs. KO2 control (black bars); **p < 0.05 vs. PDTC 3 mg/kg group; $^{\#\#}p$ < 0.05 vs. PDTC 10 mg/kg group; ***p < 0.05 vs. PDTC 30 mg/kg group, (ANOVA followed by Tukey's t test)].

Fig. 2. KO_2 induces overt pain-like behavior in a NF-κB-dependent manner. Mice were treated with PDTC (100 mg/kg, 100 µL, s.c.) or vehicle (saline, 100 µL, s.c.) 60 min before the potassium superoxide (KO_2) stimuli. The overt pain-like behavior of writhings (A) was evaluated immediately after the KO_2 (1 mg, 150 µL, i.p.) or saline (150 µL, i.p.) injection, for 20 min. In another experiment, the number of paw flinches (B), and the time spent licking (C) the injected paw were evaluated immediately after the injection of KO_2 (30 µg, 20 µL, i.pl.), for 30 min. Results are expressed as means ± SEM, n = 6 mice per group [*p < 0.05 vs. saline control; $^{\#}p$ < 0.05 vs. KO_2 control (black bars), (one-way ANOVA followed by Tukey's t test)].

Fig. 3. KO_2-induced leukocyte recruitment in the peritoneal cavity depends on NF-κB activation. Mice were treated with PDTC (100 mg/kg, 100 µL, s.c.) or vehicle (saline, 100 µL, s.c.) 60 min before the potassium superoxide (KO_2) stimulus. The peritoneal cells were harvested 4 h after injection of KO_2 (30 µg, 20 µL, i.p.) or saline (20 µL, i.p.). Total (A) and differential (B and C) cell counts were determined in samples. Results are expressed as means ± SEM, n = 6 mice per group [*p < 0.05 vs. saline control; $^{\#}p$ < 0.05 vs. KO_2 control (black bars), (one-way ANOVA followed by Tukey's t test)].

Fig. 4. KO_2-induced oxidative stress depends on NF-κB activation. Mice were treated with PDTC (100 mg/kg, 100 µL, s.c.) or vehicle (saline, 100 µL, s.c.) 60 min before the potassium superoxide (KO_2, 30 µg, 20 µL, i.pl.) or saline (20 µL, i.pl.) injection. Tissue samples from the plantar skin (A) and spinal cord (B) were collected 3 h after the KO_2 or saline injection, and the GSH levels were determined in samples by colorimetric assay. Results are expressed as means ± SEM, n = 6 mice per group [*p < 0.05 vs. saline control; $^{\#}p$ < 0.05 vs. KO_2 control (black bars), (one-way ANOVA followed by Tukey's t test)].

Fig. 5. NF-κB inhibition of KO_2-induced cytokine production. Mice were treated with PDTC (100 mg/kg, 100 µL, s.c.) or vehicle (saline, 100 µL, s.c.) 60 min before the potassium superoxide (KO_2, 30 µg, 20 µL, i.pl.) or saline (20 µL, i.pl.) injection. Tissue samples from the plantar skin (A, C and E) and spinal cord (B, D and F) were collected 3 h after the KO_2 or saline injection, and the cytokine levels were measured by ELISA. Results are expressed as means ± SEM, n = 6 mice per group [*$p < 0.05$ vs. saline control; $^{\#}p < 0.05$ vs. KO_2 control (black bars), (one-way ANOVA followed by Tukey's t test)].

Fig. 6. Superoxide anion induces IκBα degradation. Mice were treated with PDTC (100 mg/kg, 100 µL, s.c.) or vehicle (saline, 100 µL, s.c.) 60 min before the KO_2 (30 µg, 20 µL, i.pl.) or saline (20 µL, i.pl.) injection. Tissue samples from the plantar skin were collected 0.5 – 3 h after the KO_2 or saline injection, and the IκBα levels were measured by western blotting. Results are expressed as means ± SEM, n = 6 mice per group [*$p < 0.05$ vs. negative control; $^{\#}p < 0.05$ vs. KO_2 0.5 and 1 h positive controls; **$p < 0.05$ vs. KO_2 positive controls (equivalent time), (one-way ANOVA followed by Tukey's t test)].

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