UNIVERSIDADE ESTADUAL DE LONDRINA

FABIANE YURI YAMACITA BORIN

AVALIAÇÃO DA PARTICIPAÇÃO DO FATOR DE NECROSE TUMORAL (TNF) - A NA HIPERALGESIA E INFLAMAÇÃO INDUZIDAS POR DOADOR DE ÂNION SUPERÓXIDO

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Dissertação de mestrado apresentada ao Programa de Pós-Graduação em Ciências da Saúde, Centro de Ciências da Saúde, Universidade Estadual de Londrina, como requisito parcial para a obtenção do título de Mestre.

Orientadora: Profa. Dra. Rúbia Casagrande.
Co-orientador: Prof. Dr. Waldiceu Ap. Verri Jr.

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Londrina, 13 de dezembro de 2013.

Dedico este trabalho à Yutaka Yamacita,
meu pai, a quem segui na escolha da profissão,
farmacêutico apaixonado e dedicado.

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“De tudo ficaram três coisas:

A certeza de que estamos sempre começando.

A certeza de que precisamos continuar.

A certeza de que seremos interrompidos

antes de terminar. Portanto, devemos:

fazer da interrupção, um caminho novo.

Da queda, um passo de dança.

Do medo, uma escada.

Do sonho, uma ponte.

Da procura, um encontro.”

Fernando Pessoa

YAMACITA, Fabiane Yuri Borin. **Avaliação da participação do Fator de Necrose Tumoral (TNF)– α na hiperalgesia e inflamação induzidas por doador de ânion superóxido.** 2013. 82 p. Dissertação de Mestrado (Pós-Graduação em Ciências da Saúde). Universidade Estadual de Londrina, Londrina, PR, 2013.

RESUMO

Após a lesão tecidual, células residentes são ativadas e liberam citocinas e espécies reativas de oxigênio que iniciam a resposta inflamatória. Estas citocinas e espécies reativas de oxigênio desempenham papel essencial no desenvolvimento e na manutenção da dor inflamatória e do estresse oxidativo, bem como em outros eventos inflamatórios deletérios. O Fator de Necrose Tumoral (TNF)- α é uma citocina importante no desenvolvimento de dor inflamatória e na produção de ânion superóxido pela ativação do complexo NADPH oxidase. Por outro lado, a contribuição do TNF-α para a inflamação e dor induzidas pelo ânion superóxido não é compreendida. No presente estudo, a participação do TNF-α e de seu receptor TNFR1 na dor e na inflamação induzidas pelo KO_2, um doador de ânion superóxido, foi avaliada em camundongos selvagens tratados com Etanercept (receptor solúvel de TNF-α) ou em camundongos deficientes em TNFR1, respectivamente. Os animais TNFR1$^{-/-}$ exibiram redução significativa dos seguintes parâmetros nas porcentagens indicadas: dor manifesta de contorções abdominais (50%), de sacudidas (59%) e (79%) de lambidas na pata, hiperalgesia mecânica (40%), hiperalgesia térmica (59%), edema de pata (32%), recrutamento de neutrófilos (95%) e de macrófagos (72%), a depleção da capacidade antioxidante em camundongos WT diminuiu em 49% no ensaio de GSH em comparação com o grupo TNFR1$^{-/-}$, FRAP diminuiu em 42% e 24% ABTS. Foram reduzidos em camundongos TNFR1$^{-/-}$ a peroxidação lipídica (98%) e (77%) produção de anion superóxido, avaliados na pata. Os animais TNFR1$^{-/-}$ exibiram redução da contagem de leucócitos totais (85%), da contagem e atividade de neutrófilos (80% e 81%) e de células mononucleares (70% e 93%) e da produção de ânion superóxido (83%) na cavidade peritoneal após o estímulo com KO_2. Confirmando a participação do TNF-α, o estímulo intraplantar e intraperitoneal com KO_2 induziu aumento significativo na produção de TNF-α em 57% e 59%, respectivamente, e o tratamento prévio (48 horas e 1 hora antes da injeção de KO_2) com Etanercept (10 mg/kg) reduziu significativamente a hiperalgesia mecânica, a hiperalgesia térmica e o edema de pata em até 77%, 50% e 85%, respectivamente, além de reduzir em 88% e 86% a atividade de MPO e NAG. O ânion superóxido induz o estresse oxidativo dependente de TNFR1. Por sua vez, o pré-tratamento com Tempol (mimético da SOD) e Apocinina (inibidor da NADPH oxidase e antioxidante) reduziram, respectivamente, hiperalgesia mecânica (79%; 68%), edema (92%; 62%), hiperalgesia térmica (59%; 64%) e atividade de MPO (62%; 51%) induzidas por TNF-α. Os resultados deste estudo demonstram que a sinalização TNF-α/TNFR1 é importante na inflamação e hiperalgesia induzidas por ânion superóxido, e que esta sinalização amplifica o estresse oxidativo provocado pelo ânion superóxido.

Palavras-chave: TNF-α. TNFR1. Ânion superóxido. Inflamação. Hiperalgesia.

YAMACITA, Fabiane Yuri Borin. **Investigation of the role of Tumor Necrosis Factor (TNF) – α in hyperalgesia and inflammation induced by superoxide anion-donor**. 2013. 82 p. Dissertation (Master's Degree Dissertation). Londrina State University, Londrina, PR., 2013.

ABSTRACT

After tissue injury, resident cells are activated and release cytokines and reactive oxygen species that initiate the inflammatory response. These cytokines and reactive oxygen species play an essential role in the development and maintenance of inflammatory pain and oxidative stress, as well as other deleterious inflammatory events. The tumor necrosis factor (TNF) - α is an important cytokine in the development of inflammatory pain and superoxide anion production by activation of NADPH oxidase complex. On the other hand, it remains to be determined the role of TNF-α in superoxide anion-induced inflammation and pain. In the present study, the involvement of TNF-α and its receptor TNFR1 in pain and inflammation induced by KO_2, a donor of superoxide anion, was evaluated in wild-type mice treated with Etanercept (soluble TNF receptor) or in mice deficient in TNFR1, respectively. The animals $TNFR1^{-/-}$ showed significant reduction in the following parameters at indicated percentages: overt pain-like behavior of writhing (50%), flinches (59 %) and licking in paw (79%), mechanical hyperalgesia (40%), thermal hyperalgesia (59%), paw edema (32%), recruitment of neutrophils (95%), macrophages (72%), depletion of antioxidant capacity decreased in WT mice the GSH (49%), FRAP (42%) and ABTS (24%) assay compared with $TNFR1^{-/-}$. Were reduced in $TNFR1^{-/-}$ mice the lipid peroxidation (98%) and superoxide anion production (77%), evaluated in the paw. And also decreased in $TNFR1^{-/-}$ mice total leukocytes count (85%), in count and activity of neutrophils (80 % and 81%) and mononuclear cells (70% and 93%), and also in superoxide anion production (83%), in the peritoneal cavity after stimulation with KO_2. Confirming the involvement of TNF-α, intraperitoneal and intraplantar injection of KO_2 induced a significant increase in TNF-α production of 57% and 59%, respectively, and pre-treatment (48 hours and 1 hour before injection of KO_2) with Etanercept (10 mg/kg) significantly reduced mechanical hyperalgesia, thermal hyperalgesia and paw edema up to 77 %, 50% and 85%, respectively, and reduced by 88% and 86% of MPO and NAG activities. Superoxide anion induced further oxidative stress dependent on TNFR1. In turn, the pre-treatment with Tempol (SOD mimetic) and Apocynin (NADPH oxidase inhibitor and antioxidant) reduced, respectively, mechanical hyperalgesia (79%, 68%), edema (92%, 62%), thermal hyperalgesia (59%, 64%) and MPO activity (62%, 51%) induced by TNF-α. The results of this study demonstrate that signaling pathway of TNF-α/TNFR1 is important in inflammation and hyperalgesia induced by superoxide anion, and that this signaling amplifies the oxidative stress caused by superoxide anion.

Key words: TNF-α. TNFR1. Superoxide anion. Inflammation. Hyperalgesia.

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LISTA DE ABREVIATURAS E SIGLAS

ABTS	2,2- aninobis (3-etilbenzotiazolina-6-sulfonato, sal de diamônio)
Cg	Carragenina
COX	Ciclooxigenase
CXC	Quimiocina
ERN	Espécies Reativas de Nitrogênio
ERO	Espécies Reativas de Oxigênio
GSH	Glutationa reduzida
ICAM	Molécula de Adesão Intracelular
IL	Interleucina
Inos	Óxido nítrico sintase induzível
i.pl	Intraplantar
i.p	Intraperitoneal
KO_2	Superóxido de Potássio
LPO	Lipoperoxidação lipídica
LPS	Lipopolissacarídeo
MPO	Mieloperoxidase
NADPH	Fosfato de Dinucleotídio de Nicotinamida e Adenina
NAG	N-acetilglicosaminidase
NBT	Nitroazul de Tetrazólio
NFκB	Fator nuclear kapa B
NO	Óxido Nítrico
O_2^-	Ânion Superóxido
$ONOO^-$	Peroxinitrito
PGE_2	Prostaglandina E_2
SOD	Superóxido Dismutase
TBARS	Ácido tiobarbitúrico
TNF	Fator de Necrose Tumoral

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1 INTRODUÇÃO

1.1 Inflamação

A dor é um dos sinais clínicos da inflamação e é definida como experiência sensorial e emocional desagradável, em geral associada à lesão tecidual (Associação Internacional para o Estudo da Dor - IASP). A hiperalgesia de origem inflamatória resulta basicamente da interação entre o tecido danificado e os neurônios sensoriais nociceptivos periféricos (VERRI, CUNHA et al. 2006).

A ativação dos neurônios nociceptivos ocorre devido a uma propagação do potencial de ação nos terminais pré-sinápticos, onde é liberado o transmissor ou os neurotransmissores. O estímulo que causa lesão tecidual pode ativar, de forma direta ou indireta, os nociceptores, o neurônio sensorial envolvido na transmissão da informação a ser interpretada como dolorosa. A fibra nervosa inclui os nervos aferentes e eferentes, e a velocidade com que a fibra nervosa conduz o potencial de ação está relacionada com seu diâmetro, e pode ocorrer em milissegundos (FEIN, 2012).

A hiperalgesia consiste basicamente na sensibilização do neurônio nociceptivo primário através da redução de seu limiar de ativação e aumento na resposta a um estímulo doloroso. A liberação de diferentes mediadores hiperalgésicos ocorre sequencialmente durante o curso temporal da inflamação. Os chamados mediadores hiperalgésicos intermediários não atuam diretamente nas fibras nociceptivas, mas induzem a síntese de outros mediadores, chamados mediadores hiperalgésicos finais que, então, atuam diretamente nos neurônios através de receptores específicos. As citocinas são mediadores hiperalgésicos intermediários produzidos rapidamente por diversos tipos de células em resposta a uma variedade de estímulos. A produção desses e de outros mediadores, como as espécies reativas, induzem vias de sinalização responsáveis pela produção dos mediadores hiperalgésicos finais, para os quais os neurônios nociceptivos expressam receptores e assim podem ativar diretamente estes nociceptores. As citocinas constituem um elo entre a lesão celular ou reconhecimento do agente agressor, desencadeando no local os sinais e sintomas da inflamação. Os primeiros sinais da resposta inflamatória são o calor, o rubor e o edema, decorrentes da vasodilatação, do recrutamento de territórios vasculares adicionais e do extravasamento de plasma pelo território venular, além de dor e perda da função (CUNHA, VERRI et al. 2005).

Diversos mediadores podem contribuir para esta sensibilização dos nociceptores, incluindo a liberação de prostaglandinas (PGE_2) e aminas simpáticas, as citocinas interleucina (IL)-1β e o fator de necrose tumoral (TNF)-α, componentes do sistema complemento (C5a e C3a) e as espécies reativas de oxigênio (ERO) e nitrogênio (ERN),

provenientes de uma grande variedade de células (HATTORI, SUBRAMANIAN et al., 2010). Existe também evidência de que o papel das citocinas na produção de mediadores finais é precedida pelo recrutamento de neutrófilos. Assim, as citocinas são produzidas e liberadas inicialmente por células locais e, posteriormente, por leucócitos recrutados em resposta a variados estímulos inflamatórios (CUNHA, VERRI et al., 2008).

Corroborando estas afirmações, Cunha e colaboradores (CUNHA, VERRI et al., 2005), demonstraram que o TNF-α participa da redução do limiar mecânico nociceptivo em ratos e camundongos após administração de estímulos inflamatórios como carragenina (Cg) e lipopolissacarídeo (LPS), iniciando cascatas de sinalização que levam à liberação de outros mediadores inflamatórios (Figura 1).

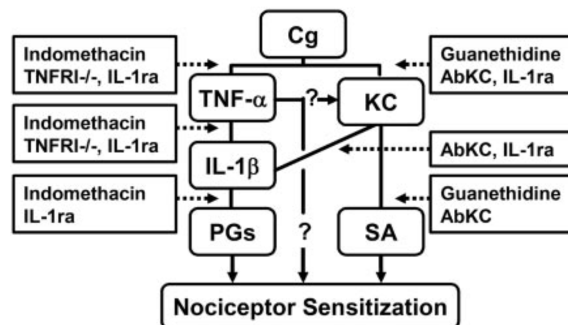

Figura 1 - Estímulo inflamatório Carragenina (Cg) induzindo a cascata de citocinas e hiperalgesia em modelo animal. O TNF-α estimula duas vias hiperalgésicas independentes que sensibilizam os nociceptores: (a) IL-1β que induz a produção de prostanóides e (b) as quimiocinas, que induzem a liberação de aminas simpáticas (CUNHA et al., 2005)

1.2 TNF-α

Conforme descrito anteriormente, após a indução da inflamação por LPS ou Cg o TNF-α é a primeira citocina a ser liberada, de modo que é considerada a citocina chave do processo inflamatório (CUNHA et al., 2005).

Atualmente o TNF-α é reconhecido como uma potente citocina pró-inflamatória, que é produzida em grandes quantidades por uma variedade de células, principalmente por macrófagos, linfócitos T CD4+, neutrófilos, células *natural killer*, mastócitos, células endoteliais, cardiomiócitos, adipócitos, fibroblastos e neurônios em resposta a estímulos inflamatórios como, por exemplo, infecções bacterianas. Os efeitos do TNF-α durante a inflamação incluem hiperalgesia, edema e recrutamento de leucócitos (BRADLEY, 2008).

O TNF-α é primariamente produzido como uma proteína transmembrana do tipo 2 estruturada em homotrímeros estáveis (memTNF) (KRIEGLER, PEREZ et al., 1988; TANG,

HUNG et al., 1996). A partir desta forma integrada à membrana forma-se a citocina homotrimérica solúvel (sTNF), que é liberada via clivagem proteolítica pela metaloproteinase enzima conversora de TNF-α (ECAT) (BLACK, RAUCH et al., 1997).

O complexo TNF-α-receptor exerce sua ação via molécula transdutora de sinal p38 MAPK, aumentando a condutividade de canais de sódio dependente de voltagem (KRAYCHETE, SAKATA, 2011). Existem dois tipos de receptor para o TNF-α, TNFR1 e TNFR2, sendo o TNFR1 considerado mais importante para a hiperalgesia inflamatória, pois induz a migração de neutrófilos, a quimiotaxia e a dor neuropática após uma injúria, enquanto o TNFR2 medeia a apoptose e a necrose celular (CHEN & GOEDDEL, 2002; RAMESH, REEVES, 2003). A interação do TNF-α com receptor TNFR1 ativa várias vias de transdução de sinal, conforme indicado na Figura 2. Anteriormente Cunha e colaboradores (CUNHA et al., 1992) investigaram o papel do TNF-α no desenvolvimento da hiperalgesia inflamatória, e posteriormente Cunha e colaboradores (CUNHA et al., 2005) usaram modelo com camundongos TNFR1$^{-/-}$ e confirmaram a participação deste receptor no efeito hiperalgésico do TNF-α.

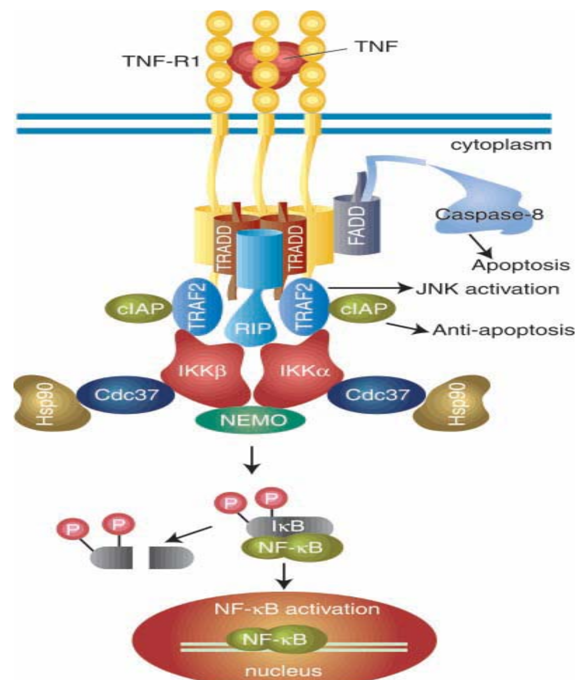

Figura 2 - A interação do TNF com receptor TNFR1 ativa várias vias de transdução de sinal (CHEN & GOEDDEL 2002).

A sinalização celular induzida pela ligação do TNF-α com TNFR1 leva a uma série de respostas, incluindo ativação de vias (p. ex. MAP quinases) que levam à ativação de fatores de transcrição, como o fator nuclear *kappa*-B (NF-κB) e c-Jun (CHEN, CAO et al., 2002; CHEN & GOEDDEL, 2002) (Figura 2).

Na sinalização por TNF-α/TNFR1, o complexo IKKβ/NEMO parece ser mais crítico. Durante a inflamação, a fosforilação de IκBα resulta na liberação dos heterodímeros de NF-κB p50/p65, permitindo a translocação dos mesmos para o núcleo da célula, induzindo a ativação transcricional de grande variedade de genes relacionados à inflamação como, por exemplo, genes para citocinas pró-inflamatórias (TNF-α, IL-1β e IL-6) e subunidades da NADPH oxidase. Como resultado há produção de mediadores inflamatórios, de ânion superóxido e de outras ERO. Também ocorre aumento na atividade das enzimas ciclo-oxigenase-2 (COX-2) e oxido nítrico sintase induzível (iNOS) (OECKINGHAUS, HAYDEN et al., 2011; HSU, LIEN et al., 2013) (Figura 2).

1.3 Etanercept

O papel significativo do TNF-α em mediar a liberação de citocinas, a inflamação tecidual e a hiperalgesia, é consistente com o efeito terapêutico de terapias anti-TNF em doenças inflamatórias crônicas como a artrite reumatoide (RANKIN, CHOY et al., 1995; TAYLOR, 2001).

Atualmente, terapias baseadas na utilização de compostos que inibem o TNF-α, como o Etanercept são eficazes em reduzir a hiperalgesia térmica, mecânica e inflamação (SOMMER, LINDENLAUB et al., 2001). Etanercept é um modulador biológico da inflamação, é uma versão recombinante humana do receptor p75 solúvel de TNF- α, que está ligada ao receptor Fc da imunoglobulina G (Ig) subclasse 1. Ele atua como um inibidor competitivo da ligação de TNF-α para os receptores de TNF da superfície celular e, assim, inibe a indução de TNF-α na atividade pró-inflamatória. Assim, Etanercept exerce ação antagonista de TNF-α (GOLDENBERG, 1999).

Corroborando esta informação, foi demonstrado que o pré-tratamento com anticorpo anti-TNF-α impede a redução do limiar nociceptivo em resposta à Cg e ao LPS, e que o tratamento com indometacina (inibidor da COX) ou com atenolol (antagonista de receptores β adrenérgicos) inibem parcialmente a hiperalgesia. Por sua vez, o co-tratamento com essas drogas é capaz de abolir completamente os comportamentos relacionados à dor (CUNHA, POOLE et al., 1992), sugerindo desta forma um papel crucial do TNF-α em modelos experimentais de hiperalgesia mecânica induzida por Cg e LPS.

1.4 Espécies reativas

O ânion superóxido é uma espécie reativa precursora de outras espécies reativas de oxigênio (ERO) e de nitrogênio (ERN) (WANG, PORRECA et al., 2004; SALVEMINI,

LITTLE et al., 2011). As principais fontes de ERO incluem: mitocôndrias (cadeia respiratória), xantina oxidases, NADPH oxidases (NOX), mieloperoxidases (MPO) e a radiação. Devido à sua reatividade, as ERO são convertidas de uma espécie para outra, como uma cascata (Figura 3). O ânion superóxido não atravessa as membranas celulares facilmente e é altamente reativo, produzindo um efeito local e deletério às biomoléculas. A enzima superóxido dismutase (SOD) é responsável pela conversão do ânion superóxido em peróxido de hidrogênio (H_2O_2), uma ERO capaz de atravessar as membranas celulares e que pode ser neutralizada pela atividade das enzimas catalase (CAT) ou glutationa peroxidase (GUZIK et al., 2000). Por outro lado, a mieloperoxidase, presente em grandes quantidades nos neutrófilos, catalisa reações que utilizam o peróxido de hidrogênio como substrato, convertendo-o em espécies altamente reativas, como o ácido hipocloroso (BRIEGER et al., 2012). Quando o ânion superóxido não é convertido pela atividade da SOD e, ao invés disso, reage com o óxido nítrico (NO), é formado o peroxinitrito ($ONOO^-$), uma espécie reativa altamente deletéria ao organismo (Figura 3)

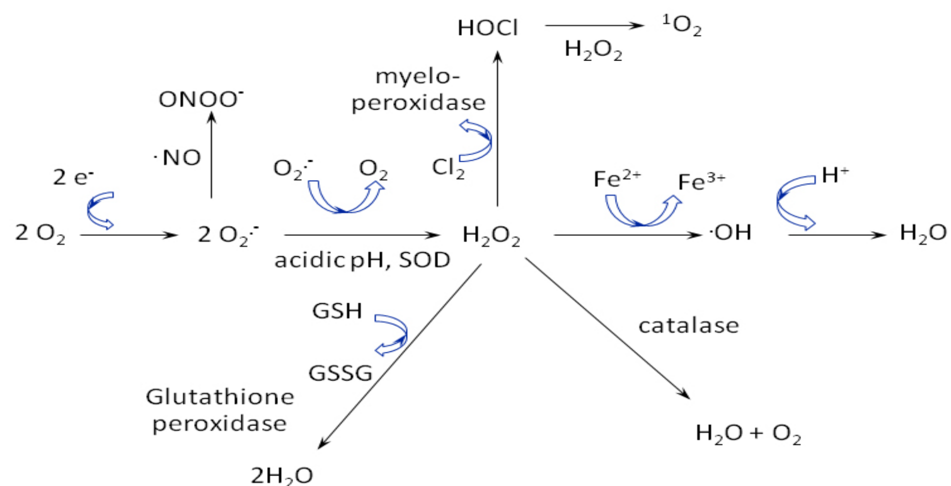

Figura 3 - Cascata da formação de ERO (BRIEGER et al., 2012).

Atualmente, o papel de ERO no contexto de saúde e doença tem sido amplamente investigado, pois em condições normais e em baixas concentrações as ERO têm importante papel em processos como: regulação da fosforilação de proteínas, canais iônicos e transcrição de fatores, produção de hormônios da tireoide e reticulação da matriz extracelular. Neste sentido, a importância das ERO pode ser observada em pacientes acometidos pela doença granulomatosa crônica (deficiência na NOX2), os quais são altamente suscetíveis aos processos infecciosos. Apesar de apresentarem importante função na defesa contra patógenos, na sinalização celular e em outros processos

fisiológicos, as ERO podem causar lesões irreversíveis e mutações ao organismo e, dessa forma, intensificar ou até mesmo desencadear diversas doenças. A exposição prolongada às altas concentrações de ERO pode levar a danos não específicos em proteínas, lipídios e ácidos nucleicos, podendo assim levar ao desenvolvimento de câncer, doenças cardiovasculares e neurodegenerativas. Os antioxidantes nem sempre são suficientes para manter o equilíbrio de ERO, podendo gerar estresse oxidativo e lesão secundária (BRIEGER et al., 2012).

1.5 Estresse oxidativo e inflamação

Conforme descrito anteriormente, a produção de espécies reativas em muitas doenças representa um importante mecanismo de lesão celular e tecidual. Durante a inflamação aguda e crônica, o ânion superóxido é produzido a uma taxa que supera a capacidade endógena do sistema antioxidante de defesa, os efeitos desse desequilíbrio foram demonstrados em diversos modelos animais (FRIDOVICH 1999; MUSCOLI, CUZZOCREA et al., 2003).

Durante o processo inflamatório, a NOX2 é ativada e produz o ânion superóxido. O ânion superóxido pode induzir a ativação de fatores de transcrição, como NF-κB, resultando na produção de citocinas pró-inflamatórias, incluindo TNF-α (Figura 4) (SCHENK, KLEIN et al., 1994; WANG, PORRECA et al., 2004). O TNF-α é uma das moléculas responsáveis pela ativação da NADPH oxidase em fagócitos durante a inflamação (KILPATRICK, SUN et al., 2010). Assim, ERO e, mais especificamente, o ânion superóxido, são importantes mediadores na hiperalgesia, edema e recrutamento de leucócitos durante a inflamação com ações periféricas e espinais (CUNHA, VERRI et al., 2005).

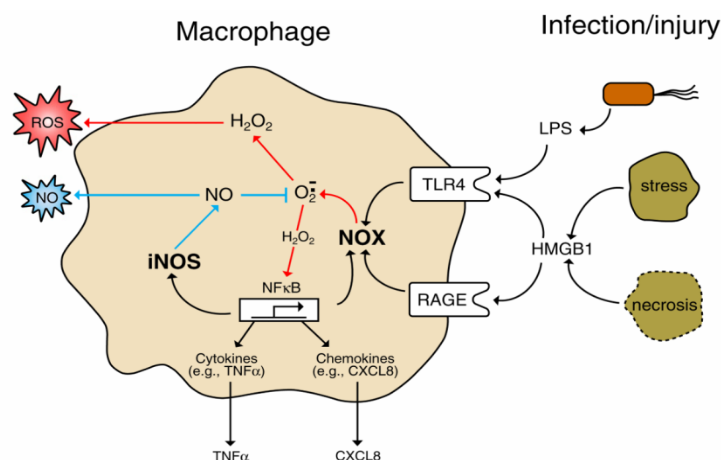

Figura 4 - Ativação de macrófago e estresse oxidativo. (KVIETYS, GRANGER, 2012)

Muitas evidências sugerem que o ânion superóxido, produzido na inflamação principalmente por neutrófilos e macrófagos, tem um papel fundamental na dor inflamatória induzida por diversos agentes (JANES, NEUMANN et al., 2012). Os neutrófilos são componentes celulares importantes do processo inflamatório agudo que, após serem ativados, produzem e liberam grandes quantidades de ânion superóxido (WANG, PORRECA et al., 2004). Contudo, o efeito do ânion superóxido tem sido demonstrado indiretamente através de compostos doadores inespecíficos de espécies reativas (como SIN-1), inibidores de vias relacionadas à superóxido dismutase (SOD) ou mesmo miméticos dessa enzima. Para avaliar diretamente os efeitos inflamatórios do ânion superóxido, nosso grupo padronizou um modelo de inflamação induzida pela injeção de superóxido de potássio KO_2, um conhecido doador de ânion superóxido (CUNNINGHAM, LOKESH, 1983). Neste modelo, o ânion superóxido induz inflamação (edema da pata, recrutamento de leucócitos, dor manifesta e hiperalgesia) e estresse oxidativo, que são reduzidas com tratamento com antioxidante quercetina (MAIOLI et al., dados não publicados).

1.6 Inibidores do Estresse Oxidativo

Substâncias antioxidantes podem ser definidas como compostos que protegem os sistemas biológicos contra os efeitos deletérios dos processos ou das reações que levam à oxidação de macromoléculas ou estruturas celulares. A produção de ânion superóxido é catalisada através de uma variedade de enzimas, incluindo a xantina oxidase, citocromo P450, lipoxigenase, enzimas da cadeia respiratória mitocondrial e NADPH oxidases (MORA-PALE et al., 2009). A glutationa peroxidase (GPx), catalase (CAT) e superóxido dismutase (SOD) são exemplos de antioxidantes enzimáticos (JORDÃO et al., 1998).

A glutationa (GSH, L-g-glutamil-L-cistenilglicina) é um tripeptídeo, contendo cisteína e é o tiol não protéico mais abundante nas células dos mamíferos. Concentrações baixas de GSH têm sido reportadas em algumas doenças que estão associadas ao estresse oxidativo. Este decréscimo do GSH pode refletir no aumento na produção de antioxidantes, num grau que excederia a capacidade de detoxificação do GSH (BRAY, 1993; MEISTER 1995). O GSSG (glutationa oxidada) é reduzido pela glutationa redutase, regenerando o GSH, num processo que depende da NADPH (KRETZSCHMAR, 1996). Para prevenir a peroxidação lipídica o GSH é usado como substrato pela glutationa peroxidase e pode através da glutationa-S-transferase detoxificar aldeídos reativos (como o malondialdeído) que são gerados durante a peroxidação lipídica. O GSH pode ser perdido de modo irreversível em situações de estresse oxidativo muito intenso, permanecendo na forma oxidada e não sendo novamente reduzido (UHLIG S & WENDEL, 1992).

A Apocinina (4-hidroxi-3-metoxiacetofenona), composto pertencente à classe dos metóxi-catecóis, foi descrita pela primeira vez em 1883 por Schmiedeberg quando foi isolada das raízes da planta *Apocynum cannabinum.* No presente estudo foi realizado um pré-tratamento com a Apocinina que pode atuar como um inibidor da ativação do complexo multienzimático NADPH oxidase (t HART, et al., 1990, LAFEBER et al., 1999, ZHANG et al., 2005, COSTA et al., 2009).

Mora-Pale e colaboradores (MORA-PALE et al., 2009) demonstraram que a oxidação enzimática da apocinina resulta na formação de um grande número de polímeros que são capazes de inibir diretamente a formação do complexo das NADPH oxidases. Neste trabalho, os polímeros resultantes da oxidação da apocinina foram capazes de inibir a interação entre duas subunidades da NADPH oxidase, p47phox e p22phox. Dessa forma, os resultados anteriores que descrevem a eficácia de apocinina como um inibidor das NADPH oxidases podem ser uma consequência de sua conversão em metabólitos ativos, provavelmente catalisada pela mieloperoxidase (MPO), enzima necessária para o efeito inibitório da apocinina sobre as NADPH oxidases. Isso sugere que a apocinina pode ser considerada uma pró-droga, uma vez que a inibição das NADPH oxidases ocorre somente após sua conversão em quinonas reativas pela atividade das MPO. Essas quinonas, por sua vez, se ligam aos resíduos de Cys na sub-unidade p47phox e interrompem a translocação para a membrana. Uma vez que a MPO é secretada em grandes quantidades pelos neutrófilos ativados e, dessa forma, está presente em grande quantidade nos tecidos durante o processo inflamatório, a inibição das NADPH oxidases pelos produtos da oxidação da apocinina pode ocorrer mesmo em células que não expressem MPO. Dessa forma, apesar de alguns estudos relacionarem os efeitos protetores da apocinina com sua atividade antioxidante, principalmente em células que não contém MPO (HEUMULLER et al., 2007), a presença de neutrófilos no contexto inflamatório parece suprir a ausência de MPO e gerar produtos capazes de inibir diretamente as NADPH oxidases nessas células.

Por outro lado, Petrônio e colaboradores (2013) ressaltaram a capacidade antioxidante da apocinina como um mecanismo direto para alguns dos seus efeitos biológicos, sendo eficaz para espécies como HOCl e H_2O_2.

O Tempol (4-hydroxy- 2,2,6,6-tetramethylpiperidine-N-oxyl) é um mimético da enzima superóxido dismutase (SOD) e sua função é metabolizar o ânion superóxido à peróxido de hidrogênio, que por sua vez sofre ação da catalase formando H_2O e O_2^-. Assim, o Tempol pode interromper a formação do ânion superóxido e diminuir a inflamação e hiperalgesia induzidas por espécies reativas de oxigênio (KHATTAB, 2006).

Os efeitos prejudiciais do peroxinitrito são mediadas através de diferentes mecanismos, incluindo a peroxidação lipídica e inativação enzimática (CORVO E BECKMAN, 1995), a depleção de glutationa (PHELPS et al., 1995) e danos ao DNA (INOUE

& KAWANISHI, 1995). A multiplicidade do efeito citotóxico de peroxinitrito pode denotar que poderia ser uma das espécies reativas mais nocivas em inflamação aguda, tal como demonstrado pelo edema induzido por carragenina na pata (CUNHA et al., 2005). A interrupção da formação de peroxinitrito por meio de inibição da formação do ânion superóxido, através da utilização de um mimético da SOD, o TEMPOL, pode ser eficiente na inflamação aguda peroxinitrito-dependente. Usando um mimético da SOD, o M40403, Wang et al. (2004) demonstraram o fundamental papel do ânion superóxido na dor inflamatória, pois reduziu a hiperalgesia inflamatória induzida por carragenina em ratos através da diminuição da produção de citocinas, tais como TNF-α hiperalgésicos.

E no estudo de Khattab (2006) destacou-se o potencial efeito anti-inflamatório do mimético da SOD em inflamação aguda, sugerindo que o efeito anti-inflamatório do mesmo, não é mediado apenas pela inibição do ânion superóxido, mas também por prevenir a resposta inflamatória do tecido pela formação do peroxinitrito.

1.7 Justificativa

As espécies reativas de oxigênio apresentam papéis como moléculas que induzem lesão celular, por outro lado, sua função na sinalização celular também é extremamente importante. O papel de espécies reativas de oxigênio no desenvolvimento de dor e inflamação deve ser melhor investigado para aumentar a compreensão das suas funções, das conseqüências da sua inibição e sua interação com outras moléculas envolvidas na inflamação e dor. Dentre as moléculas com relação com o ânion superóxido destaca-se o TNF-α e seu receptor TNFR1 devido ao seu papel na inflamação e dor e de ser um alvo clínico consolidado em doenças como a artrite reumatóide. O TNF-α induz a ativação da NADPH oxidase e produção do ânion superóxido. Porém, ainda falta compreender a via inversa, ou seja, da possível participação do TNF-α e TNFR1 na inflamação e dor induzidos pelo ânion superóxido.

2 OBJETIVOS

2.1 Objetivo Geral

Verificar a participação e os mecanismos que relacionam o TNF-α e seu receptor TNFR1 com os processos inflamatórios e nociceptivos induzidos pelo ânion superóxido.

2.2 Objetivos Específicos

- Avaliar se o doador de ânion superóxido induz a produção de TNF-α;

- Avaliar se os animais deficientes para TNFR1 apresentam redução na inflamação e na dor inflamatória induzida pelo ânion superóxido;

- Avaliar se a inibição do TNF-α pela administração do Etanercept reduz a hiperalgesia e inflamação induzidas pelo doador de ânion superóxido;

- Avaliar se a inibição do estresse oxidativo com Tempol e Apocinina reduzem a hiperalgesia e inflamação induzidas pelo TNF-α.

3 MATERIAS E MÉTODOS

3.1 Animais experimentais

Os experimentos foram realizados utilizando camundongos da linhagem C57BL/6 (20 a 25g) normais ou deficientes (-/-) para o receptor tipo 1 do TNF-α (TNFR1). Os camundongos C57BL/6 e $TNFR1^{-/-}$ foram fornecidos pelo biotério da Faculdade de Medicina de Ribeirão Preto - USP de Ribeirão Preto. Para realização dos experimentos foram selecionados grupos de 06 camundongos, inteiramente ao acaso. Os camundongos ficaram no biotério em ciclo claro/escuro (12h/12h), com livre acesso a água e ração. As amostras selecionadas tinham características similares, sendo da mesma raça, mesmo sexo, mesma idade e pesos próximos. Foram realizadas duas repetições para cada tratamento, até três repetições quando foi necessário. O número de animais por grupo (n=6) foi o utilizado na literatura e mínimo para a análise estatística.

Todos os experimentos foram conduzidos de acordo com as normas estabelecidas pelo Comitê de Ética em Experimento Animal da Universidade Estadual de Londrina, CEUA-UEL n° 71.2012.68.

3.2 Protocolos Experimentais e Tratamento

Os estímulos inflamatórios (TNF-α e doador de ânion superóxido - KO_2) foram administrados por via subcutânea intraplantar ou intraperitoneal (VERRI et al., 2007). As doses administradas de KO_2 foram 30 μg em solução salina via intraplantar (i.pl.) e dose de 1000 μg por via intraperitoneal (i.p.) para avaliação da dor manifesta nas contorções abdominais nos grupos normal e $TNFR1^{-/-}$, doses estas padronizadas previamente pelo laboratório (MAIOLI et al., dados não publicados). Animais inoculados somente com solução salina foram utilizados como controle. O estímulo com TNF foi na dose de 100 pg/ pata (CUNHA et al., 2005).

Os experimentos de hiperalgesia mecânica, térmica e edema foram avaliados nos intervalos de tempo 0, 0,5, 1, 3 e 5 horas. Os camundongos foram eutanasiados com isofluorano inalatório e a atividade de mieloperoxidase (MPO) e N-acetilglicosaminidase (NAG) na pata foram avaliadas na quinta hora; a avaliação da migração de leucócitos na cavidade peritoneal e contagem de células na sexta hora; estresse oxidativo e dosagem de TNF- α na terceira hora.

Foi realizado pré-tratamento com Etanercept (Enbrel® 50mg) via i.p. 48 horas e 1 hora antes da administração do estímulo KO_2 em determinado experimento nas doses de 1mg/Kg; 3mg/Kg e 10mg/Kg diluídos em solução salina, um grupo sem tratamento prévio e

com administração de KO_2 e um grupo apenas com solução salina foram avaliados simultaneamente no experimento.

Em outro experimento foi realizado tratamento prévio com Apocinina 30mg/Kg (i.p.) e Tempol 100mg/Kg (i.p.) diluídos em solução salina, 1 hora antes da administração do estímulo TNF-α (i.pl.) nos camundongos WT. Um grupo sem tratamento prévio e com administração de TNF-α e um grupo apenas com solução salina foram avaliados simultaneamente no experimento.

3.3 Dor manifesta

A avaliação da dor manifesta foi realizada através do teste do número de contorções abdominais. O estímulo foi injetado na cavidade peritoneal do animal, e logo após cada animal foi colocado num cilindro de vidro grande, um por vez, sendo que a intensidade do comportamento nociceptivo foi quantificada através da contagem do número total de contorções abdominais ocorridas entre 0 e 20 minutos após a administração do estímulo. A resposta de contorções abdominais consiste de uma contração do músculo abdominal, juntamente com um alongamento de membros posteriores (MAIOLI et al., dados não publicados).

No teste do número de sacudidas e tempo de lambida os animais foram colocados em compartimentos de vidro transparente, um por vez, após a administração intraplantar do estímulo. O número de elevações e o tempo gasto lambendo a pata foram determinados entre 0 e 30 minutos após a administração do estímulo (MAIOLI et al., não publicado).

3.4 Hiperalgesia mecânica

A avaliação da hiperalgesia mecânica foi realizada com analgesímetro com filamentos de Von Frey (Frey, 1896), (Modelo 1601C, Life Science Instruments). Os animais foram colocados em gaiolas de acrílico 15 minutos antes da primeira medição. O analgesímetro eletrônico consiste em um transdutor de pressão adaptado a um contador digital de força expressa em gramas (g), onde o contato do transdutor de pressão com a pata do camundongo foi realizado através de uma ponta descartável de polipropileno. Foi realizada uma medição antes da administração do estímulo, ou seja, no tempo zero, e posteriormente após administração intraplantar do estímulo e medição nos tempos

determinados. Foi considerada a média de três medições. Os resultados foram relatados como o delta (Δ) da força (*g*), sendo calculado subtraindo o valor das medições após o estímulo do tempo zero. A intensidade de hiperalgesia foi quantificada como a variação na pressão (Δ de reação em gramas) obtida (CUNHA, VERRI et al., 2004).

3.5 Hiperalgesia térmica

A hiperalgesia induzida por estímulo térmico foi avaliada de acordo com o método descrito previamente (VERRI, MOLINA et al., 2005). Para este experimento, os camundongos foram transferidos para placa de metal mantida por uma fonte de calor a 55°C. A resposta ao estimulo térmico foi avaliada pelo comportamento de sacudidas da pata, sapatiado ou lambida. O tempo máximo para permanência na placa quente foi de 30 s para evitar lesão tecidual (TRIBUTINO, SANTOS et al., 2010; LI, ZHANG et al., 2013).

3.6 Edema

Para avaliação do edema induzido pelo KO_2 e TNF-α, a espessura da pata foi medida com auxílio de um paquímetro (Digmatic Caliper, Mitutoyo Corporation, Kanagawa, Japan). O edema foi calculado como a diferença entre as medidas das patas injetadas no tempo zero (sem estímulo) e medida das patas dos grupos experimentais nos diferentes intervalos de tempo após a administração do estímulo.

3.7 Recrutamento de leucócitos

Foi realizada a administração do estímulo na cavidade intraperitoneal dos animais. Após 6 horas da adiminstração, os animais foram eutanasiados com isofluorano, e as células foram coletadas da cavidade intraperitoneal por lavagem com 2 mL de tampão fosfato. A contagem do número total de células foi realizada em câmara de Neubauer em solução de Turk. A contagem diferencial foi realizada pelo preparo de esfregaços, os quais foram corados pela técnica de Panótico rápido e as células diferenciadas em microscópio óptico através da objetiva em óleo de imersão (aumento de 100x). Os resultados foram apresentados como o número de células por cavidade (VERRI, CUNHA et al., 2007).

3.8 Atividade da mieloperoxidase (MPO)

O ensaio cinético-colorimétrico da enzima mieloperoxidase (MPO) é um método indireto, utilizado para avaliar a migração de neutrófilos para o tecido subcutâneo plantar das patas traseira e do peritôneo (CASAGRANDE, GEORGETTI et al., 2006). Após 5 horas

da administração do estímulo, os camundongos foram eutanasiados com isofluorano e amostras de tecido subcutâneo plantar foram colhidos em 50 mM de K2HPO4 tampão (pH 6,0) contendo Brometo de hexadeciltrimetilamônio 0,5% (HTAB) e mantida a -80 ° C até à utilização. As amostras foram homogeneizadas utilizando um Polytron (PT3100), sendo centrifugadas a 16.100 g, 4°C, durante 2 minutos, e o sobrenadante resultante utilizado para o ensaio da atividade de MPO. As absorvâncias das amostras foram registradas em espectrofotômetro de microplacas (ThermoScientific, MultskanGO) em comprimento de onda de 450 nm, com três leituras em 1 min. A atividade da MPO das amostras foi comparado com uma curva padrão de neutrófilos. Os resultados foram apresentados como a atividade de MPO, ou seja, número de neutrófilos por mg de tecido.

3.9 Atividade da N- acetilglicosaminidase (NAG)

A avaliação da atividade do NAG (N-acetilglicosaminidase) é um ensaio enzimático em que se avalia de forma indireta a quantidade de macrófagos presente no local. Para o ensaio de atividade de NAG, após 5 horas da administração do estímulo, os camundongos foram eutanaziados com isofluorano e amostras de tecido subcutâneo plantar foram colhidos. Após 20 ul de sobrenadante foram colocados numa placa de 96 poços, seguidos pela adição de 80 mL de K2HPO4 50 mM (pH 6,0). A reação foi iniciada pela adição de 2,24 mM de 4-nitrofenilo N-acetil-β-D-glucosaminidase, incubados a 37 ° C durante 10 minutos, e interrompida por adição de 100 mL de 0,2 M de tampão de glicina (pH 10,6). A atividade de NAG foi determinada espectrofotometricamente a 400 nm (Multiskan GO ThermoScientific). A absorvância das amostras foi comparada com uma curva padrão de macrófagos e apresentado como actividade NAG (número de macrófagos × 10^4/mg de tecido).

3.10 Dosagem de TNF-α

Para o teste de dosagem de citocinas, foi avaliada a quantidade de TNF-α no tecido plantar e na cavidade intraperitoneal dos grupos salina e no grupo com estímulo. As amostras de tecido plantar foram coletadas 3 horas após o estímulo com KO_2 (30 μg) e homogeneizadas (Polytron®) em tampão para dosagem de TNF- α utilizando kits comerciais de ELISA de acordo com as normas do fabricante (eBioscience, Ready-SET-Go).

3.11 Avaliação do estresse oxidativo

O estresse oxidativo foi avaliado através do Teste da Glutationa (GSH), Teste do Poder Antioxidante pela Redução do Ferro (FRAP) e Teste do ABTS para avaliar a

capacidade antioxidante da amostra (HOHMANN, CARDOSO et al. 2013). Teste do ácido tiobarbitúrico (TBARS) que avalia a peroxidação lipídica (BARBOSA, CECCHINI et al. 2003) e para avaliar a produção do ânion superóxido foi realizado o ensaio de tetrazólio nitroazul (NBT).

3.11.1 Teste da Glutationa (GSH)

Os níveis de GSH foram determinadas utilizando um ensaio de fluorescência. Após administração do estímulo, a pele da pata dos animais foram reunidas e homogeneizadas em 100 mM de NaH2PO4 (pH 8,0) contendo 5 mM de EGTA utilizando um Polytron (PT3100). Após foi tratada com 30% de ácido tricloroacético e centrifugada 1940g durante 6 min. O sobrenadante foi utilizado para o ensaio de fluorescência. A curva padrão foi preparada com 0-40 iM de GSH, e os resultados foram apresentados como nM de GSH por mg de pele (VALÉRIO et al, 2009).

3.11.2 Teste do Poder Antioxidante pela Redução do Ferro (FRAP)

O ensaio de FRAP mede a mudança na absorvância a 593 nm devido à formação de um composto de cor azul oxidado pela doação de elétrons antioxidantes. Foi utilizada 1,5 mL do reagente de trabalho FRAP que foi aquecido até 37 °C e uma leitura do reagente em branco foi realizada em 593 nm. Subsequentemente, 50 µL da amostra de tecido da pata e 150 µL de água desionizada foram adicionadas ao reagente FRAP e essa mistura foi incubada a 37 °C por 4 minutos. Foi realizada a leitura da absorvância após os 4 minutos de incubação. A diferença entre a leitura do branco e a leitura contendo a amostra foi calculada e utilizada para o cálculo do valor de FRAP com referência no resultado que foi obtido por uma solução de FeII de concentração conhecida. Os valores foram expressos como micromole de FeII por grama de tecido. Todas as medições foram feitas em quatro repetições para cada amostra (HOHMANN, CARDOSO et al. 2013).

3.11.3 Teste do ABTS

Este ensaio é baseado na inibição da absorvância do radical catiônico 2,2 V-azinobis 3-etilbenzotiazolino 6-sulfonato (ABTS), que se da pelos radicais livres presentes na amostra. Esse radical tem espectro de absorção máxima a 734 e 820 nm. Para o estudo, a solução ABTSS + foi diluída com tampão fosfato pH 7,4 (PBS) a uma absorvância de 734 nm e equilibrada a 30°C. Foi realizada a medição do ABTS sem amostra, ou seja, o branco, e após a medição do ABTS contendo a amostra. A amostra foi preparada com 2,0 mL de

ABTS solução diluída e 20 mL da amostra de tecido da pata. Também foi realizada a medição com Trolox padrão. As amostras foram incubadas durante 6 minutos à 30°C e a absorvância foi determinada exatamente aos 6 minutos após incubação para todas as amostras. Todas as medições foram realizadas em quatro repetições. A porcentagem de inibição do ABTS + pela amostra foi calculado de acordo com a fórmula:

$$\% \text{ Inibição} = [(Abs_{Branco} - Abs_{amostra}) / Abs_{Branco}] \times 100$$

A capacidade de eliminação de radicais livres da amostra foi calculado como porcentagem de inibição de ABTS + equiparado contra uma curva padrão de Trolox. Os resultados foram expressos como Trolox equivalentes por grama de peso de tecido (HOHMANN, CARDOSO et al. 2013).

3.11.4 Ensaio TBARS

O conteúdo de proteína de homogenato (10 mg / ml em 1,15% de KCl), foi medido utilizando o método de Lowry. Substâncias que reagem ao ácido tiobarbitúrico (TBARS) medida foi utilizada para avaliar a peroxidação lipídica (LPO). Para este ensaio, o ácido tricloroacético (10%) foi adicionado ao homogenato para precipitar as proteínas. Esta mistura foi então centrifugada (3 min, 1000 g). A amostra livre de proteína foi extraída e de ácido tiobarbitúrico (0,67%) foi adicionado. A mistura foi mantida em banho-maria a 100 °C, 15 min. Malondialdeído (MDA), um produto intermediário da lipoperoxidação, foi determinada pela diferença entre as absorvâncias a 535 e 572 nm num espectrofotómetro leitor de microplacas (Multiskan GO, Thermo Scientific) e os resultados foram relatados em nmol / mg de proteína.

3.11.5 Produção de O_2^- – ensaio do Nitroazul de Tetrazólio (NBT)

A quantificação da produção de O_2^- no homogenato de tecido (10 mg / ml em 1,15% de KCl) foi realizada utilizando o ensaio de tetrazólio nitroazul (NBT) (CHOI, KIM et al. 2006). Incubar 50 uL de homogeneizado com 100 ul de NBT (1 mg / ml) em placas de 96 poços a 37° C durante 1h. O sobrenadante foi então cuidadosamente removido e o formazano solubilizado reduzida pela adição de 120 uL de 2 M de KOH e 140 uL de DMSO. A redução do NBT foi medido a 600 nm utilizando um espectrofotómetro leitor de microplacas (Multiskan GO, Thermo Scientific). O teor de proteínas foi utilizado para a normalização dos dados. Os valores foram ajustados utilizando os pesos das amostras (mg de tecido) e os dados apresentados como redução do NBT a 600 nm.

3.12 Análise Estatística

A análise estatística foi realizada verificando a normalidade dos resultados pelo teste de Shapiro-Wilk e a homogeneidade de variâncias analisado pelo teste de Levene. Os resultados obtidos foram expressos como média ± erro padrão da média (EPM). Os dados foram comparados estatisticamente utilizando a análise de variância (ANOVA) seguido do teste de Tukey ou de Student, usando o software GraphPad Prism 5. Em todos os cálculos foi fixado o nível de significância de 5% ($p < 0,05$).

4 RESULTADOS E DISCUSSÃO

Os resultados do atual trabalho estão descritos no artigo científico a ser submetido à revista Free Radical Research com o título "*TNF-α/TNFR1 signaling mediates the inflammation and inflammatory pain induced by superoxide anion in mice*". As normas da revista encontram-se no Anexo A.

4.1 Artigo

TNF-α/TNFR1 signaling mediates the inflammation and inflammatory pain induced by superoxide anion in mice

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ABSTRACT

TNF-α and superoxide anion are produced during inflammation. In the present study, the role of TNF-α and its receptor TNFR1 in potassium superoxide (superoxide anion donor) induced inflammation was investigated. The potassium superoxide-induced overt pain-like behavior (paw flinch [59%] and licking [79%]), mechanical hyperalgesia (40%), thermal hyperalgesia (59%), paw edema (39%), myeloperoxidase (95%) and N-acetyl-galactosaminidase (72%) activities, decrease of reduced gluthathione (49%), decrease of ferric reducing ability potential (42%), reduction of ABTS scavenging potential (24%), lipid peroxidation (98%), superoxide anion production (77%) were reduced in TNFR deficient mice compared to wild type mice in the paw tissue. In the peritoneal cavity, TNFR1 deficient mice also presented reduced potassium superoxide-induced abdominal writhings (50%), total leukocytes count (85%), total neutrophils (81%) and mononuclear cells (93%), and superoxide anion production (83%) compared to wilt type mice. Etanercept (soluble TNF receptor) reduced in a dose-dependent manner potassium superoxide-induced paw edema (85%), mechanical hyperalgesia (77%), thermal hyperalgesia (50%), myeloperoxidase (88%) and N-acetyl-galactosaminidase (86%) activities. Potassium superoxide also increased TNF-α levels in the paw and peritoneal cavity by 57% and

59% respectively. In turn, the pre-treatment with Tempol and Apocynin reduced mechanical hyperalgesia (79%, 68%), edema (92%, 62%), thermal hyperalgesia (59%, 64%) and MPO activity (62%,51%) induced by TNF-α, respectively. These data demonstrate that TNF-α/TNFR1 signaling is important in superoxide anion-triggered inflammation and nociception, and that TNF-α/TNFR1 signaling amplifies the oxidative stress triggered by superoxide anion.

Introduction

During the inflammatory process, reactive oxygen species (ROS) are produced in large amounts by cellular activation. TNF-α is an important cytokine that induces the production of ROS such as superoxide anion ($O_2^{\bullet -}$) via NADPH oxidase activation [1]. The effects of TNF-α during inflammation include hyperalgesia, edema and leukocyte recruitment [2-4]. The hyperalgesic effect of TNF-α in acute inflammatory hyperalgesia depends on further production of nociceptive molecules, and targeting such molecules reduce the hyperalgesia induced by TNF-α [5].

In addition to the microbicidal role of ROS, they also present important signaling roles. For instance, ROS such as superoxide anion induce the activation of the transcription factors NFκB and AP-1 resulting in the production of pro-inflammatory cytokines including TNF-α [6-8].

Moreover, superoxide anion is a precursor of many other ROS such as hydroxyl and peroxynitrite, which are more deleterious than superoxide anion itself. The evidence on the role of superoxide anion in inflammation and pain is indirect

since the majority of studies used generators of peroxynitrite, a product of superoxide anion reaction with nitric oxide (NO), or mimetics of superoxide dismutase (SOD), reducing the levels of superoxide anion [6,8,9]. In fact, the SOD mimetic Tempol reduces the increased paw edema and thermal hyperalgesia induced by peroxynitrite [10]. The treatment with the SOD mimetic M40403 reduces carrageenin-induced inflammatory hyperalgesia in rats by diminishing the production of hyperalgesic cytokines such as TNF-α [8]. Thus, indicating that targeting superoxide anion reduces inflammation and hyperalgesia, and that a positive feedback loop might exist between superoxide anion and TNF-α. Considering the

clinical relevance of TNF-α-targeting therapies in inflammatory diseases [11], it is important to understand its effects and mechanisms as well as its interaction with other molecules involved in inflammation to improve patient treatment.

To evaluate the inflammatory effects of superoxide anion, we have standardized a model with the injection of potassium superoxide [13], a well known superoxide anion donor [12]. In this model, superoxide anion induces inflammation (paw edema, leukocyte recruitment, overt pain-like behavior and hyperalgesia) and oxidative stress, which are amenable by treatment with antioxidant quercetin. Furthermore, the nociceptive behavior elicited by potassium superoxide is reduced by treatment with the opioid, morphine, confirming that a nociceptive response is under evaluation [13].

Taking into account the above mentioned evidence, the role of TNF-α/TNFR1 in superoxide anion-induced inflammation and pain was investigated in mice.

Materials and Methods

Animals

Adult male C57BL/6 and TNFR1$^{-/-}$ mice from University of São Paulo, São Paulo, Brazil, were used in this study (n=6). Mice were housed in standard clear plastic cages with free access to food and water, with a light/dark cycle of 12:12 h, at 21 °C. All behavioral tests were performed between 9 a.m. and 5 p.m. in a temperature-controlled room. The mice were used only once. 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, Londrina, Brazil (process 71.2012.68). All efforts were made to minimize the number of animals used and their suffering.

Drugs and reagents

Etanercept was purchased from Amgen Inc. (Thousand Oaks, CA, USA). 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, trichloroacetic acid (TCA), thiobarbituric acid (TBA), nitro blue tetrazolium (NBT), HTAB (Bromide, hexadecyltrimethylammonium), dihydrochloride *orto*-dianisidine, glutathione (GSH), EDTA sodium salt, ferric chloride hexahydrate, TPTZ (2,4,6-tripiridil-s-triazine), ABTS [2,2-azinobis (3-ethylbenzothiazoline-6-10 sulfonate), diammonium salt], Trolox (6-hydroxy-2 ,5,7,8-tetramethylchroman-2-11 carboxylic acid), Tempol, and Apocynin were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Experimental protocols

In the overt pain-like behavior tests, the number of paw flinches and time spent licking the paw were determined between 0-30 min after intraplantar (i.pl.) injection of 30 µg of KO_2 per animal (in 25 µL of sterile saline), and the number of writhings was measured between 0-20 min after intraperitoneal (i.p.) injection of 1 mg of KO_2 per animal (in 100 µL of sterile saline). Mechanical hyperalgesia, thermal hyperalgesia and paw edema were evaluated 0.5, 1, 3 and 5 h after i.pl. injection of KO_2 (30 µg/paw), and the samples from plantar tissue were collected immediately after the last measurement and used in both MPO and NAG assays. In order to investigate the role of TNF-α in KO_2-induced hyperalgesia and edema, mice were treated with Etanercept (1, 3 or 10 mg/kg, i.p.) 48 hours and 1 hour before i.pl. stimuli with KO_2. The TNF-α levels were also measured in plantar tissue and peritoneal exudates samples 3 h after KO_2 stimulus.

In another approach, the role of TNF-α/TNFR1 signaling pathway in leukocyte recruitment was investigated in peritoneal cavity. The KO_2 was administered by intraperitoneal (i.p.) route at the previously described dose of 30 µg per cavity (in 100 µL of sterile saline). After 6 h, mice were euthanized with isofluorane and the peritoneal cavities were washed with 200 µL PBS containing EDTA (37.2 mg/100 mL saline). The samples from peritoneal exudates were then used in leukocyte counts, MPO, NAG and NBT assays.

Furthermore, the redox status of plantar tissue samples were also measured in samples collected 3 h after KO_2 stimulus using ABTS, FRAP, NBT and TBARS methods as previously described [14,15]. All intervals of measurement and doses of KO_2 that were used in this study were obtained from previous study [13]. Tempol and apocynin treatments were administered by i.p. route 1 hour before KO_2 stimulus.

Electronic Pressure-meter Test

The test consisted of evoking a hindpaw flexion reflex with a hand-held force transducer (Modelo 1601C, Life Science Instruments) adapted with a 0.5 mm^2 polypropylene tip. Detailed methodology was previously described by our group [16]. The results are expressed by delta (Δ) withdrawal threshold (in *g*) calculated by subtracting the zero-time mean measurements from the mean measurements at the indicated time points after stimulus.

Hot Plate Test

The test was performed as previously reported [17]. In brief, mice were placed in a 10 cm wide glass cylinder on a hot plate (Hot Plate HP-2002, Insight Equipamentos, Ribeirão Preto, SP, Brazil) maintained at 55°C. The reaction time was scored when the animal jumped or licked its paws. The normal latency (reaction time) was 15-30s. The reaction time was scored when the animal jumped or licked its paws. A maximum latency (cut-off) was set at 30 s to avoid tissue damage. Respective control groups were injected i.pl. with the same volume of sterile saline

Writhing Response Tests

Each mouse was placed in a large glass cylinder and the intensity of nociceptive behaviour was quantified by counting the total number of writhings occurred between 0-20 min after i.p. injection of KO_2 [13]. The intensity of the writhing response was expressed as the cumulative writhing score.

Paw flinches and Time spent licking Test

The number of paw flinches and the time spent licking the paw were determined between 0-30 min after i.pl. injection of KO_2. Results were expressed as the cumulative number of flinches and the time spent licking behaviours observed in ipsilateral paw.

Paw edema

Paw edema was measured with caliper (Digmatic Caliper, Mitutoyo Corporation, Kanagawa, Japan). Values of paw thickness are expressed in millimetres (Δ mm) as the difference between the diameter measured before (basal) and after i.pl. injection of KO_2.

MPO and NAG activity

The neutrophil and macrophage recruitment to the paw skin was evaluated by the MPO and NAG colorimetric assays, respectively, as previously described [15,18,19]. Briefly, mice were terminally anesthetized and the paw skin samples were collected in 400 µl of 50 mM K_2HPO_4 buffer (pH 6.0) containing 0.5% of HTAB, and then homogenized in ice-cold using Tissue-Tearor (Biospec®). After that, homogenates were centrifuged (16 100 *g*, 2 min, 4 °C) and the supernatants were collected. For MPO assay, aliquots of 30 µL of supernatant were was placed in a 96-well and mixed with 200 µL of 50 mM K_2HPO_4 buffer (pH 6.0), containing 0.0167 % *ortho*-dianisidine dihydrochloride and 0.05 % H_2O_2. The absorbance was determined after 5 min at 450 nm (Multiskan GO Microplate Spectrophotometer, ThermoScientific, Vantaa, Finland). The MPO activity of samples was compared to a standard curve of neutrophils and presented as MPO activity (number of neutrophils × 10^4/mg of tissue).

For NAG activity assay, 20 µL of supernatant were placed in a 96-well plate, followed by the addition of 80 µL of 50 mM K_2HPO_4 (pH 6.0). The reaction was initiated by the addition of 2.24 mM 4-nitrophenyl N-acetyl-β-D-glucosaminide, incubated at 37 °C for 10 min, and stopped by addition of 100 µL of 0.2 M glycine buffer (pH 10.6). The NAG activity was determined spectrophotometrically at 400 nm (Multiskan GO ThermoScientific). The absorbance of samples was compared with a standard curve of macrophages and presented as NAG activity (number of macrophages × 10^4/mg of tissue).

Leukocyte recruitment to the peritoneal cavity

Mice were terminally anesthetized 6 h after i.p. stimulus with KO_2 and the peritoneal cavities were washed with 200 µL PBS containing EDTA (37.2 mg/100 mL saline). Aliquots of 20 µL were used in total leukocytes counts after dilution in 180 µL of Turk's solution. Differential neutrophil and mononuclear cell counts were performed using the Kit Panotic Fast for histological analysis (Laborclin, Pinhais, PR, Brazil) and values are expressed as numbers of cells per cavity (x10^6). Total leucocytes and differential counts were both performed under an optical microscope using Neubauer chambers or microscope slides, respectively.

GSH, FRAP and ABTS assays

GSH levels were determined using a fluorescence assay. After administration of the stimulus, the skin of the paw of the rats were pooled and homogenized in 100 mM NaH_2PO_4 (pH 8.0) containing 5 mM EGTA using a Polytron (PT3100). After treated with 30% trichloroacetic acid and centrifuged 1940g for 6 min. The

supernatant was used for fluorescence assay. The standard curve was prepared with 0-40 uM GSH, and the results were expressed as mM of GSH per mg of skin [22].

Samples of paw skin were collected and immediately homogenized with 500 µL of 1.15% KCl buffer. The homogenates were centrifuged (200 *g*, 10 min, 4 ºC) and supernatants were used to measure the ability of samples to resist to oxidative damage (FRAP assay) and to scavenging free cationic radicals (ABTS assay), as previously reported [14]. The absorbance was measured spectrophotometrically at 595 nm for FRAP assay and 730 nm for ABTS assay (Multiskan GO ThermoScientific) and the results were equated against a Trolox standard curve (0.02-20 nmol). The results are expressed as nmol Trolox equivalent/mg of tissue weight in both assays.

Lipid Peroxidation

Lipid peroxidation in the plantar tissue was assessed by determining thiobarbituric acid reactive substances (TBARS) levels using a method previously described [15,20]. For this assay, TCA 10 % was added to the homogenate and the mixture was centrifuged (1000 *g*, 3 min, 4°C) to precipitate proteins. The protein-free supernatant was then separated and mixed with TBA (0.67%). The mixture was kept in water bath (15 min, 100 °C). Malondialdehyde (MDA), an intermediate product of lipid peroxidation, was determined by difference between absorbances at 535 and 572 nm using a microplate spectrophotometer reader. The results were presented as nmol of MDA per mg of tissue.

Superoxide Anion Production

The superoxide anion production was determined by NBT reduction method as previously described [15]. Briefly, the plantar skin samples were homogenized with 500 µL of saline, and aliquots of 50 µL of homogenate were incubated with 100 µl of NBT solution (1 mg/mL) in a 96-well plate for 1 h at 37 °C. The supernatants were carefully removed, and the formazan precipitated was then solubilized by adding 120 µL of 2 M KOH and 140 µL of DMSO. The optical density was measured at 600 nm (Multiskan GO ThermoScientific). The weight of samples was used for data normalization, and results were presented as NBT reduction (OD/mg of tissue).

TNF-α Levels

The TNF-α levels were measured in samples of plantar tissue and peritoneal exudate. The samples were collected mice from euthanized with isoflurane and immediately kept at -80 ° C. Frozen plantar tissue samples were homogenized in 500 µL of saline in an ice bath. Both samples were centrifuged (800 *g*, 10 min, 4 °C), and the resulting supernatants were collected. The TNF-α level on supernatant was determined by ELISA sandwich assay kit, according to the manufacturer's instructions (eBioscience). Results were presented as pg of TNF-α per 100 mg of tissue or as pg of TNF-α per cavity.

Statistical Analysis

Results are presented as means ± SEM of measurements made on 6 mice in each group. One way or Two way ANOVA followed by Tukey's *t*-test or Student were performed to compare the values at the indicated time points. Statistical differences were considered to be significant when $P < 0.05$.

Results

Superoxide induces overt pain-like behavior depends on TNFR1 activation

KO_2 significantly induced the overt pain-like behaviors of writhing score and flinching/ licking on the wild type (WT) mice. There was a reduction of 50% in writhing score (Figure 1A), 59% in flinching (Figure 1B) and 79% in time spend licking the paw (Figure 1C) in TNFR1 deficient ($TNFR1^{-/-}$) mice compared with WT mice.

TNFR1 deficiency reduces of superoxide-induced mechanical and thermal hyperalgesia and edema

Superoxide or saline was injected in WT and $TNFR1^{-/-}$ mice, and the mechanical hyperalgesia (Figure 2A), paw edema (Figure 2B) and thermal hyperalgesia (Figure 2C) were evaluated 0.5 - 5h after superoxide. Superoxide induces mechanical hyperalgesia, edema and thermal hyperalgesia that was reduced in $TNFR1^{-/-}$ mice in 40%, 32% and 59%, respectively. Superoxide increases the mieloperoxidase and NAG activity, and it was reduced in $TNFR1^{-/-}$ mice in 95% (Figure 2D) and 72% (Figure 2E).

TNF-α mediates inflammatory pain induced by superoxide anion.

In order to confirm the role of TNF-α in superoxide-induced inflammation and pain, mice were pre-treated with Etanercept (TNF-α inhibitor on doses of 1, 3, or 10 mg/kg, i.p.) or vehicle, 48 hours and 1 hour before i.pl. stimulus with superoxide anion. The dose of 10mg/Kg of Etarnecept decreases the mechanical hyperalgesia (77%, Figure 3A), paw edema (85%, Figure 3B) and thermal hyperalgesia (50%,

Figure 3C). Furthermore, the mieloperoxidase activity was reduced in 88% (Figure 3E) and 86% on NAG activity (Figure 3F) with pre-treated with Etanercept 10mg/kg. After that, the TNF-α production on paw, 3h after the superoxide injection was increased in 57% compared with saline group (Figure 3D).

TNF-α mediates superoxide-induced leukocyte recruitment and production of superoxide anion in peritoneal cavity

Superoxide or saline was injected in WT and TNFR1$^{-/-}$ mice, and the total and differential leucocytes were evaluated. Corroborating with the results obtained with i.pl. injection of KO_2, 6h after the stimulus TNFR1-deficient mice showed a significant reduction in total leucocytes recruitment (85%, Figure 4A), 80% in

polymorphonuclear (Figure 4B) and 70% in mononuclear leukocytes recruitment to peritoneal cavity (Figure 4C). Moreover, it was also observed that TNFR1-deficiency mice inhibits in 81% the mieloperoxidase activity (Figure 4D) and 93% on NAG activity (Figure 4E). Furthermore, the intraperitoneal injection of KO_2 induced superoxide anion production in 83% (NBT assay, Figure 4F). TNF-α level on peritoneal lavage, 3h after the superoxide injection was increased in 59% compared with saline group (Figure 4G). in fact, TNF-α is also important mediator in peritoneal inflammatory processes.

Superoxide-induced oxidative stress depends on TNFR1 activity.

The redox status of plantar tissue samples from TNFR1-deficient mice was measured and compared with samples from WT mice (Figure 5). First, the antioxidant status of samples was measured by electron transfer-related methods, in

WT mice the GSH assay decreased in 49% compared with TNFR1$^{-/-}$ and 55% compared with saline group (Figure 5A), FRAP decreased in 42% compared with TNFR1$^{-/-}$ and 45% compared with saline group (Figure 5B) and ABTS decreased 24% compared with TNFR1$^{-/-}$ and 30% compared with saline group (Figure 5C). It was observed that TNFR1$^{-/-}$ prevents the decrease in antioxidant ability that is induced by superoxide injetion. Moreover, it was also demonstrated that KO_2-induced superoxide anion production, and TNFR1-deficient mice decreased in 98% the lipid peroxidation (TBARS assay, Figure 5D) and 77% (NBT assay, Figure 5E).

The mechanical hyperalgesia, thermal hyperalgesia, and edema induced by TNF-α were inhibited by tempol and apocynin pre-treatment

The apocynin is an important inhibitor of oxidative stress by inhibition of NADPH oxidase complex, and tempol, a SOD mimetic, inhibited the superoxide anion formation. Pre-treatment with apocynin reduced in 68% the mechanical hyperalgesia (Figure 6A), 62% edema (Figure 6B) and 64% thermal hyperalgesia (Figure 6C). The pre-treatment with tempol reduced in 79% the mechanical hyperalgesia (Figure 6A), 92% edema (Figure 6B) and 59% thermal hyperalgesia (Figure 6C). The mieloperoxidase activity were also evaluated and the treatment with apocynin reverted in 51% and tempol in 62% (Figure 6D), with a significant between WT group and saline group, but no significant difference between the groups apocynin and tempol.

Discussion

The role of TNF-α on a superoxide anion-induced inflammation have not been explored, herein, we demonstrated for the first time that targeting TNF-α/TNFR1 reduced the abdominal writhings, paw flinch and licking behavior, mechanical and thermal hyperalgesia, paw edema, MPO and NAG activity, leukocyte recruitment and oxidative stress.

In a study of Maioli et al. (unpublished data) [13] it was shown that a donor of superoxide anion induces hyperalgesia and inflammation in a dose- and time-dependent manner. The present data suggest that superoxide anion-induced inflammation and pain depends on, at least in part, on further production of TNF-α, which induces additional superoxide anion production. Previously Cunha et al. (1992) [28] conducted a study that assessed the importance of TNF-α in the development of inflammatory hyperalgesia in rats. Subsequently Cunha et al. (2005) [5] evidenced the involvement of a cascade of cytokines in mediating carrageenin-induced mechanical hyperalgesia. It was found that TNF-α acting on TNFR1 is an important cytokine in inflammatory hyperalgesia, and that inflammation and hyperalgesia were reduced by treatments with anti-TNF-α. Furthermore, Borghi et al. (unpublished data) [29] demonstrated that in an experimental model of exhaustive exercise, TNF-α/TNFR1 is important to mediate the inflammation and hyperalgesia. Moreover, Sommer et al. (2001) [30] demonstrated that Etanercept reduced the hyperalgesia in an experimental model of neuropathic pain. Therefore, TNF-α/TNFR1 signaling is important in inflammatory and neuropathic pain states.

However, other molecules beyond TNF-α are involved in inflammatory hyperalgesia, and the participation of additional molecules seems also to occur in superoxide anion-induced inflammation since targeting TNF-α/TNFR1 did not abolished all the nociceptive and inflammatory responses induced by potassium

superoxide. For instance, Ribeiro et al. (2000) [26] demonstrated that the nociceptive activity of zymosan and acetic acid in the writhing response model are dependent on peritoneal resident macrophages and mast cells, and that they are activated by the release of TNF-α, interleukin 1β (IL-1β) and interleukin 8 (IL-8). Furthermore, the release of prostaglandins and sympathetic amines are secondary to the release of pro-inflammatory cytokines by resident tissue cells.

Superoxide anion induces oxidative stress during inflammation and represents an important pathway that leads to peripheral sensitization of nociceptors [21]. There is elevation of oxidative stress and ROS production during inflammatory and painful conditions as well as inhibiting the oxidative stress reduces inflammation and pain indicating an important role of ROS [21-24]. Contrasting with the importance of ROS in inflammatory pain and hyperalgesia, superoxide anion and hydrogen peroxide secondarily generated as reaction products from pyrogallol and Fe-EDTA, respectively, did not induce ongoing activity or sensitize nociceptors to mechanical and thermal stimuli in a rat skin-saphenous nerve preparation [25]. This absence of effect was also observed in the presence of inflammatory molecules such as bradykinin, histamine, serotonine and prostaglandin E_2, further indicating that superoxide anion and hydrogen peroxide do not directly activate nociceptive neurons. Nevertheless, a possibility is that superoxide anion acts in the cells surrounding the nociceptive neurons to induce the production of nociceptive molecules. Furthermore, it is likely that superoxide anion reacting with NO results in peroxynitrite, which nitrates SOD maintaining increased levels of superoxide anion that acts by inducing cytokine production [8]. Kvietys (2012) [37] demonstrated that the release of TNF-α and chemokines along with an imbalance between oxidant

species and intracellular antioxidants, occurs to amplify the inflammatory response and hyperalgesia. In agreement, a SOD mimetic, M40403, reduced inflammation and hyperalgesia [8].

The present study shows that superoxide anion donor-induced inflammation and pain depends on TNF-α/TNFR1 signaling by a positive feedback loop in which superoxide anion induces the production of TNF-α that, in turn, induces superoxide anion production. This positive feedback loop was corroborated by the present data demonstrating that superoxide anion induces TNF-α production, inflammation and pain, and that all these evaluated parameters were inhibited by Etanercept (soluble TNF receptor) and/or on TNFR1 deficient mice.

The present data lines up with previous works demonstrating that superoxide anion and TNF-α mediate similar responses including: overt pain-like behavior [8,26,27], mechanical hyperalgesia [28,38], thermal hyperalgesia [8,39], edema [8,31,32], MPO and NAG activity [32-35], total leukocyte, neutrophil and mononuclear cell recruitment [32,36,37], and oxidative stress [37]. Thus, the present data in addition to previous evidence support that the inflammatory roles of superoxide anion depends on TNF-α. Therefore, we suggest that targeting superoxide anion might represent a therapeutic approach in diseases in which inhibiting TNF-α production and/or action is clinically relevant.

Conclusions

TNF-α/TNFR1 signaling contributes significantly to potassium superoxide (superoxide anion donor) induced inflammation and pain. There is a positive feedback loop in which superoxide anion induces TNF-α production, which in turn

induces superoxide anion-mediated inflammation and pain. This study contributes to understanding the inflammatory and nociceptive interaction between superoxide anion and TNF-α suggesting that in addition to TNF-α, targeting superoxide anion is a conceivable approach to reduce inflammation.

Declaration of Interest Section

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Author contributions

Performed experiments (FYYB, ACZ, FAPR), analized and interpretated data (FYYB, ACZ, FAPR, SHF, JCAF, FQC, TMC, RC, WAVJ), experimental design and conception of study (RC, WAVJ), wrote the paper (FYYB, RC, WAVJ), approved the final version to be published (FYYB, ACZ, FAPR, SHF, JCAF, FQC, TMC, RC, WAVJ).

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Figure legends

Fig. 1 Superoxide-induced overt pain-like behavior depends on TNFR1 activatio*n*. Mice received KO_2 injection via i.p. route (1 mg, 100 μL) and the (A) writhing behavior was evaluated. In another set of experiment, mice received KO_2 i.pl. injection (30 μg/25 μL) and the (B) number of flinches and (C) time spent licking the

paw were observed. **P* < 0.05 compared with saline group, #*P* < 0.05 compared with wild type (WT) + KO_2 group. ANOVA followed by Tukey's *t* test (*n* = 6).

Fig. 2 Superoxide-induced mechanical hyperalgesia, thermal hyperalgesia, edema and plantar neutrophil and macrophage recruitment were inhibited in TNFR1-deficient mice. (A) Mechanical hyperalgesia, (B) thermal hyperalgesia, (C) edema, (D) myeloperoxidase (MPO) and (E) N-acetyl-β-D-glucosaminidase (NAG) activities were determined in samples were measured after KO_2 i.pl. stimuli (30 μg, 25 μL) at the indicated time points or equivalent volume of saline. **P* < 0.05 compared with saline group, #*P* < 0.05 compared with wild type (WT) + KO_2 group. ANOVA followed by Tukey's *t* test (*n* = 6).

Fig. 3 TNF-α mediates inflammation and inflammatory pain induced by superoxide anion. Mice were pre-treated with Etanercept (1, 3, and 10 mg/kg, i.p.) or vehicle 48 hours and 1 h before i.pl. injection of KO_2 (30 μg, 25 μL) or saline. (A) Mechanical hyperalgesia, (B) thermal hyperalgesia, (C) edema, (E) MPO activity and (F) NAG activity were measured at the indicated time points. (D) Mice received i.pl. injection with KO_2 (30 μg, 25 μL) or equivalent volume of saline, after 3 h, plantar tissue samples were collected and TNF-α levels measured by ELISA. **P* < 0.05 compared with saline group, #*P* < 0.05 compared with WT + KO_2 group. ANOVA followed by Tukey's *t* test (*n* = 6).

Fig. 4 TNF-α mediates superoxide-induced leukocyte recruitment and production of superoxide anion in peritoneal cavity through TNFR1 activation. (A) Total and (B and C) differential leukocyte counts, (D) MPO, (E) NAG, and (F) NBT reducing activity were all determined in WT and $TNFR1^{-/-}$ mice 6 h after i.p. injection of KO_2 (30 μg, 150 μL) or equivalent volume of saline. (G) Mice received i.p. injection with KO_2 (30

μg, 100 μL) or equivalent volume of saline. After 3 h, peritoneal cavities were washed with PBS and the samples were used to measure TNF-α levels by ELISA. $*P < 0.05$ compared with saline group, $^{\#}P < 0.05$ compared with wild type (WT) + KO_2 group. ANOVA followed by Tukey's *t* test ($n = 6$).

Fig. 5 Superoxide-induced oxidative stress depends on TNFR1 activity. Mice received i.pl. injection of KO_2 (30 μg, 25 μL) or equal volume of saline. After 3 h, samples from plantar tissue were collected and used in (A) GSH, (B) FRAP, (C) ABTS, (D) TBARS and (E) NBT assays. $*P < 0.05$ compared with saline group, $^{\#}P < 0.05$ compared with WT + KO_2 group. ANOVA followed by Tukey's *t* test. ($n = 6$).

Fig. 6 TNF-α-induced mechanical hyperalgesia, thermal hyperalgesia, and edema were inhibited by Tempol and Apocynin. Mice were pre-treated with Tempol or Apocynin or vehicle i.p. 1 h before i.pl. injection of TNF-α (100 pg/paw) or saline. (A) Mechanical hyperalgesia, (B) thermal hyperalgesia, (C) edema and (D) MPO activity were measured after TNF-α i.pl. stimuli (100 pg, paw) at the indicated time points. $*P < 0.05$ compared with saline group, $^{\#}P < 0.05$ compared with vehicle group. ANOVA followed by Tukey's *t* test. ($n = 6$).

Figures

Figure 1

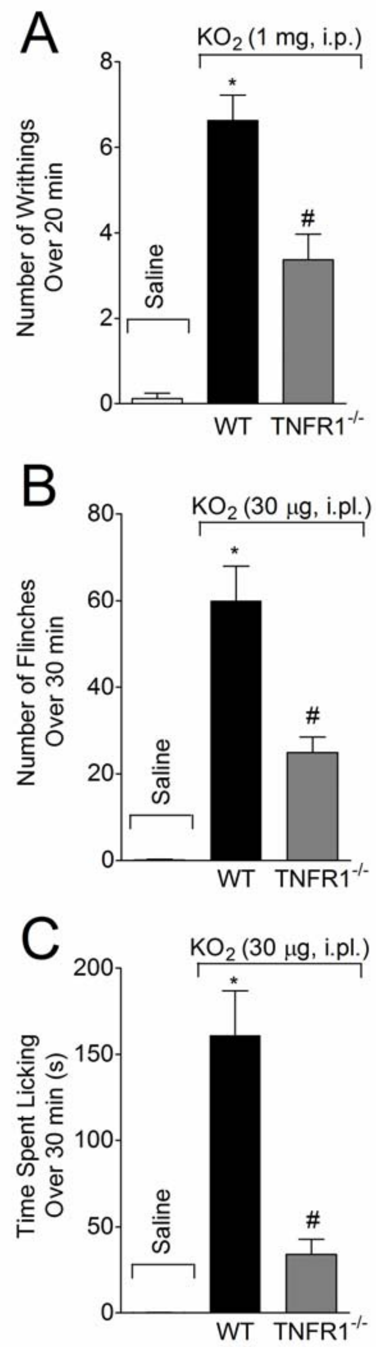


Figure 2

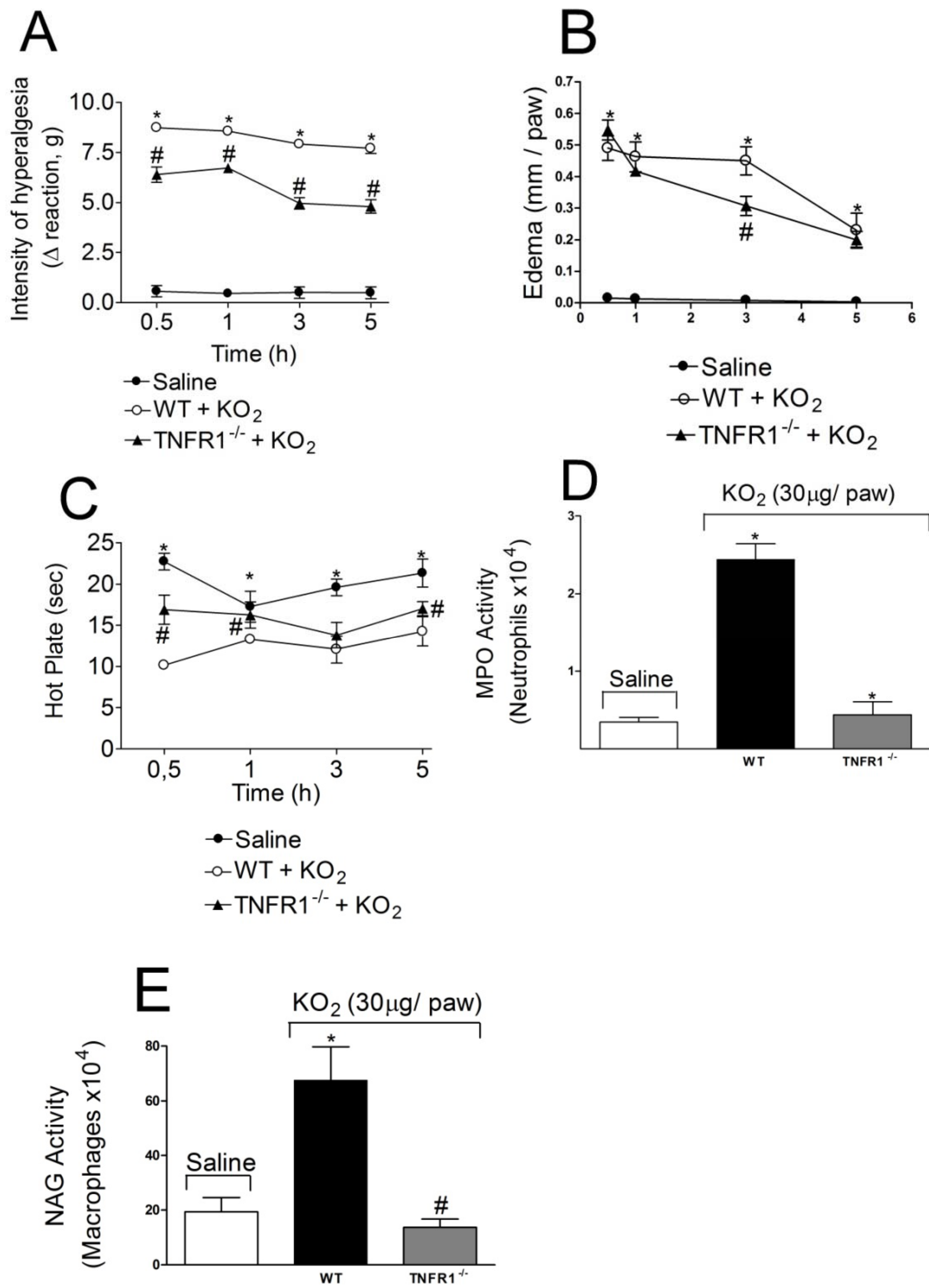
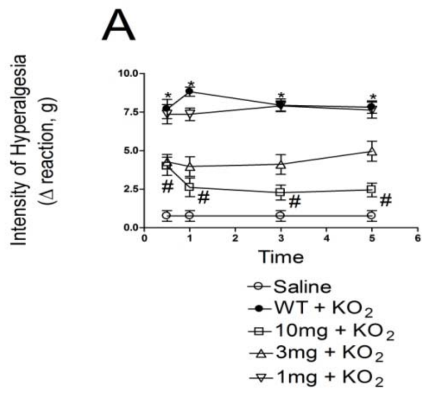
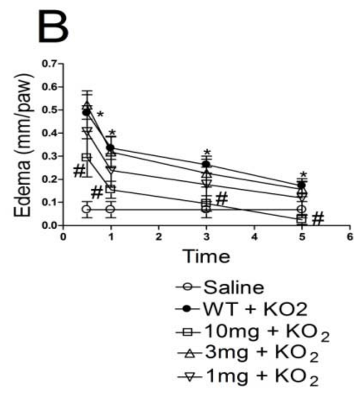
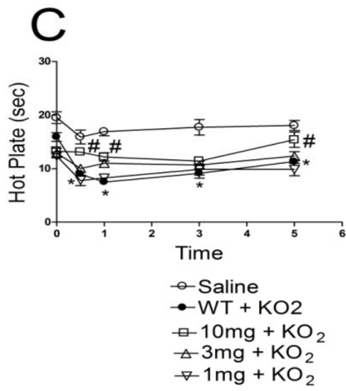
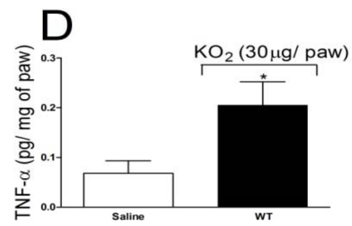


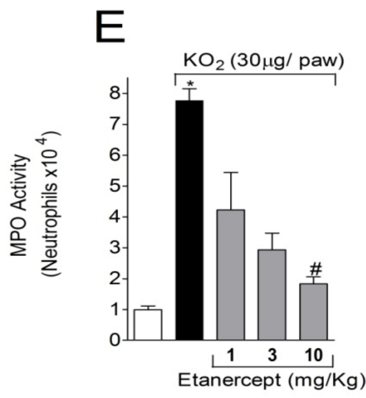
Figure 3


A
Intensity of Hyperalgesia
(Δ reaction, g)
Time
Saline
WT + KO2
10mg + KO2
3mg + KO2
1mg + KO2


B
Edema (mm/paw)
Time
Saline
WT + KO2
10mg + KO2
3mg + KO2
1mg + KO2


C
Hot Plate (sec)
Time
Saline
WT + KO2
10mg + KO2
3mg + KO2
1mg + KO2


D
TNF-α (pg/ mg of paw)
KO2 (30μg/ paw)
Saline
WT


E
MPO Activity
(Neutrophils x10 4)
KO2 (30μg/ paw)
1
3
10
Etanercept (mg/Kg)

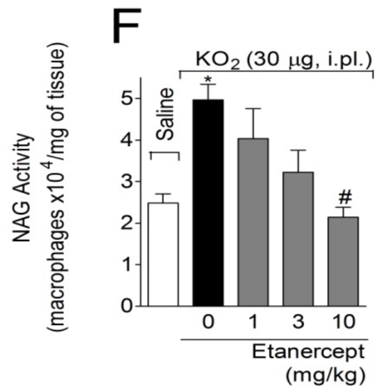

F
NAG Activity
(macrophages x10 4 /mg of tissue)
KO2 (30 μg, i.pl.)
Saline
0
1
3
10
Etanercept
(mg/kg)

Figure 4

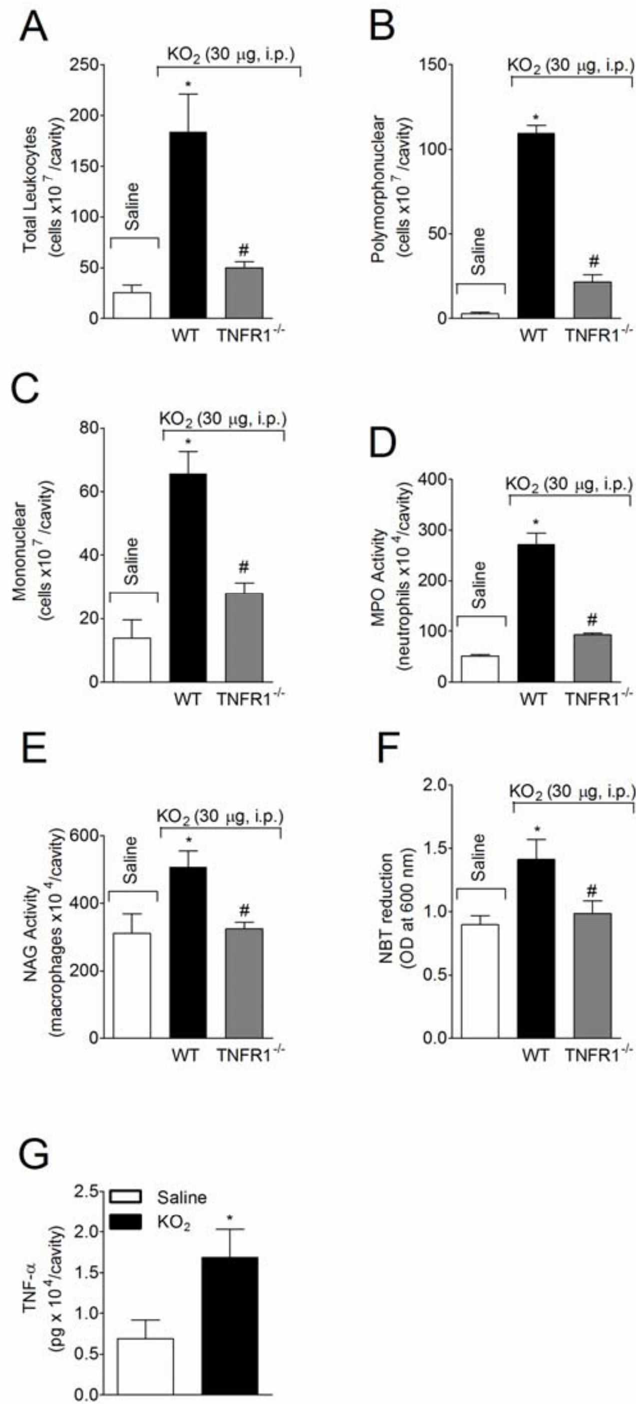


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Figure 5

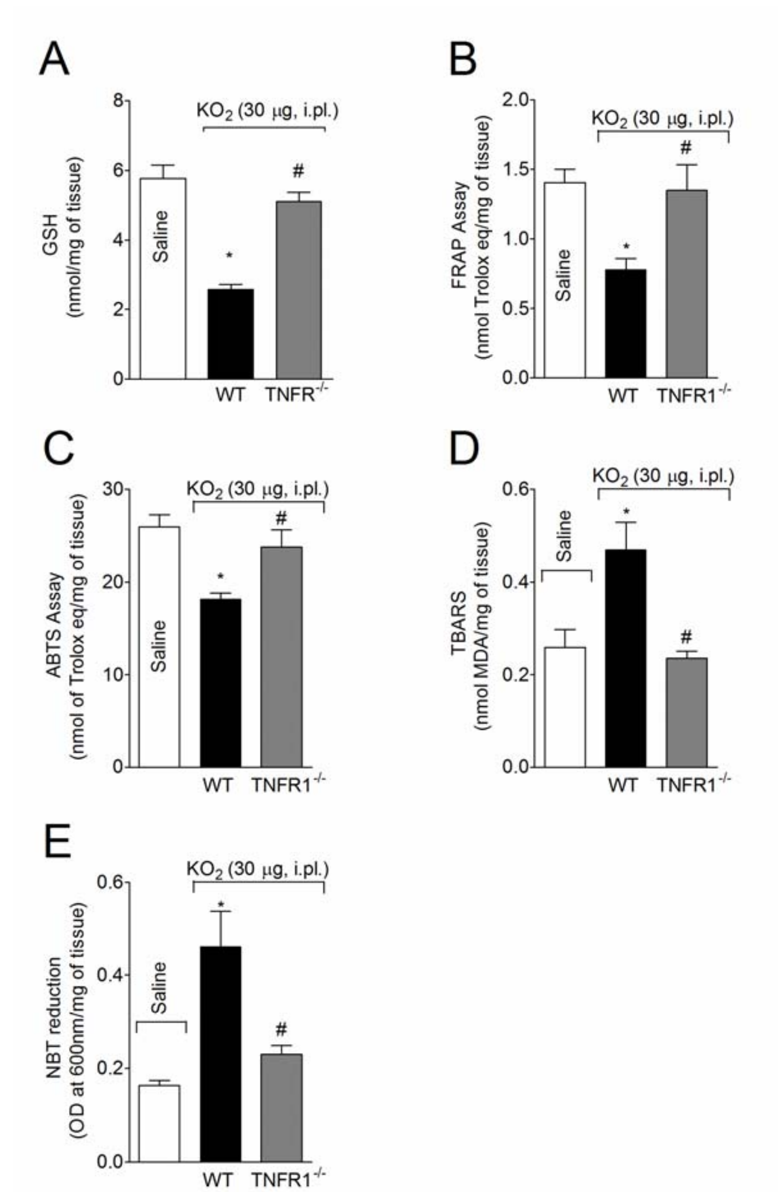
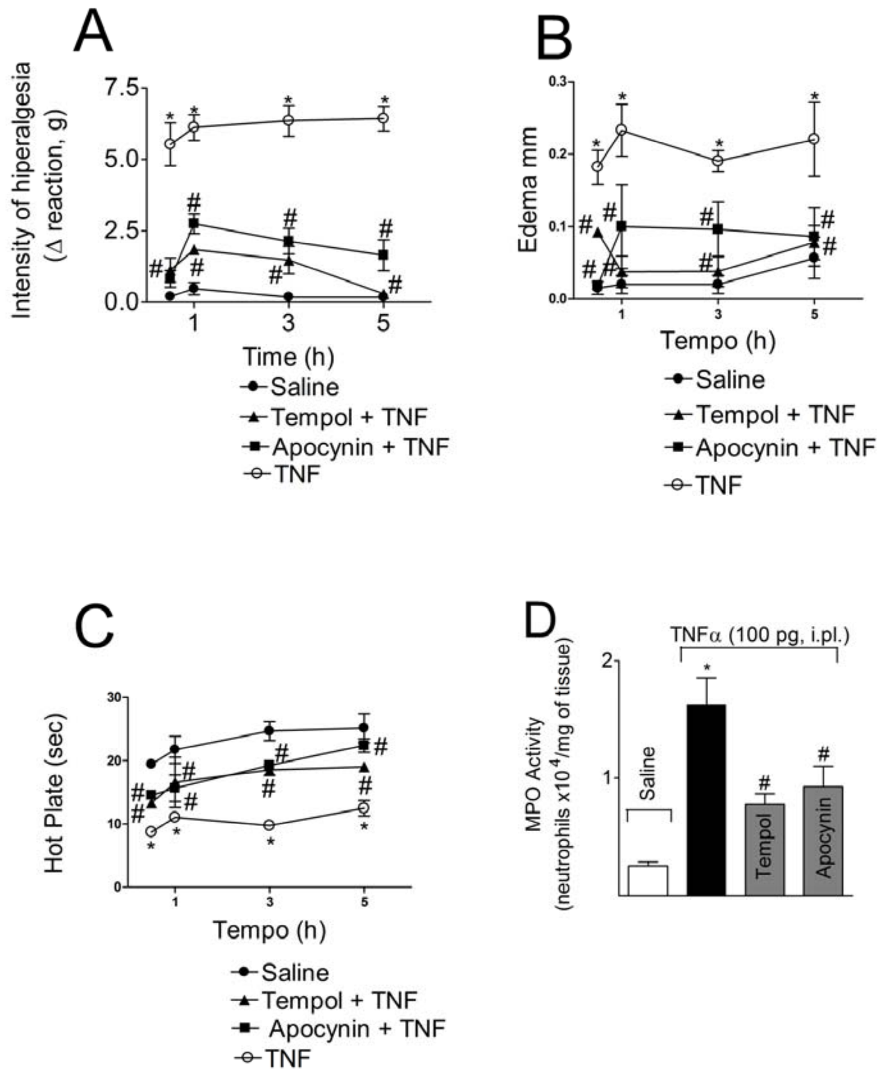


Figure 6



5 CONCLUSÕES

Foi demonstrado no presente estudo que a sinalização do TNF-α/TNFR1 contribui significativamente para a inflamação e para a dor inflamatória induzidas pelo superóxido de potássio, um doador de ânion superóxido. Há um ciclo de retroalimentação (*feedback* positivo), em que o ânion superóxido induz a produção de TNF-α, que por sua vez induz inflamação mediada pelo ânion superóxido (Figura 5). Este estudo contribui para a compreensão dos mecanismos que sustentam a resposta inflamatória e a dor resultante da atividade do ânion superóxido. A inter-dependência entre ânion superóxido e TNF-α sugere que a inibição deste ciclo pode representar um importante alvo terapêutico em doenças inflamatórias agudas e crônicas.

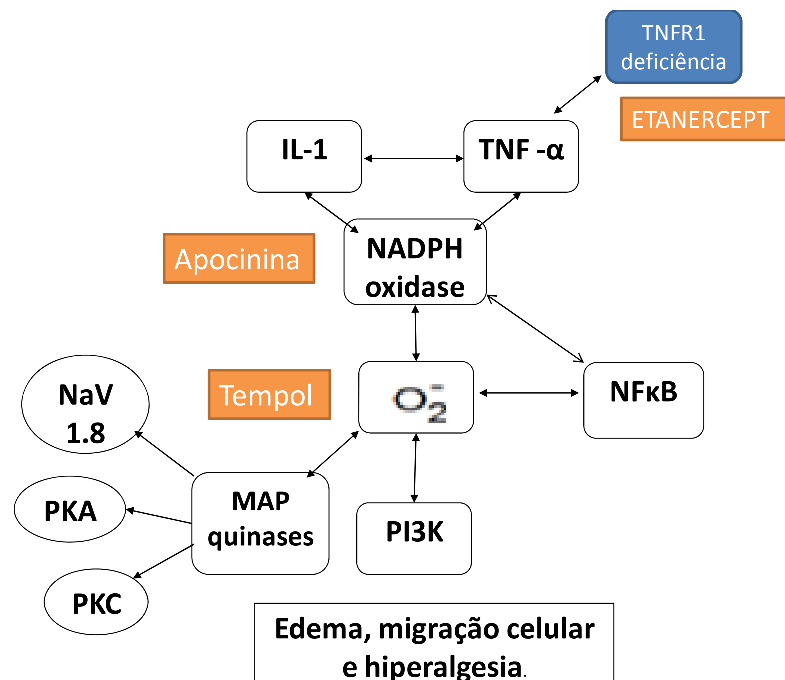


Figura 5 - Fluxograma experimental do TNF-α

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ANEXO

ANEXO A

Free Radical Research

Instructions for Authors

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- Antioxidants, including diet-derived antioxidants and other relevant aspects of human nutrition
- Oxidative damage, mechanisms and measurement

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