Universidade Estadual de Londrina

AMANDA ZAPAROLI ZUCOLOTO

AVALIAÇÃO DOS MECANISMOS ANALGÉSICOS E ANTI-INFLAMATÓRIOS DE PROBUCOL EM MODELOS DE INFLAMAÇÃO AGUDA EM CAMUNDONGOS

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Dissertação apresentada ao programa de pós-graduação em 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 Junior

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BANCA EXAMINADORA

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

_____
Profa. Dra. Andressa de Freitas Mendes Dionísio
Universidade Estadual de Londrina - UEL

_____
Profa. Dra. Graziela Scalianti Ceravolo
Universidade Estadual de Londrina - UEL

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RESUMO

Probucol é um composto polifenólico sintético clinicamente utilizado na prevenção e tratamento de doenças cardiovasculares. No entanto, estudos têm demonstrado seu efeito benéfico em modelos experimentais de outras doenças inflamatórias, como diabetes, doença de Huntington e lesão isquêmica. Dentre os possíveis mecanismos de ação da droga, já foram descritos aumento de antioxidantes endógenos e inibição de citocinas pró-inflamatórias. Estudos *in vitro* demonstraram também que probucol inibe ativação de NF-κB e expressão de moléculas de adesão envolvidas no recrutamento celular. A ativação clássica de NF-κB é crucial para produção de mediadores pró-hiperalgésicos como TNF-α, IL-6 e IL-1β em quadros de dor inflamatória. Dessa forma, a fim de investigar o potencial analgésico e anti-inflamatório de probucol, o presente trabalho utilizou modelos de inflamação aguda induzida por carragenina ou lipopolissacarídeo (LPS) e sub-aguda por adjuvante completo de Freund (CFA). A dor manifesta foi avaliada por número de contorções abdominais em 20 minutos (induzida por ácido acético ou fenil-p-benzoquinona) e número de sacudidas de pata e tempo gasto lambendo a pata em 30 minutos após estímulo por CFA. Nos modelos de inflamação aguda por carragenina ou LPS, os animais receberam probucol v.o. 1 h antes do estímulo. A hiperalgesia mecânica e térmica foram avaliadas 1, 3 e 5 h após o estímulo utilizando analgesímetro digital e placa quente, respectivamente. A atividade de N-acetilglicosamina e mieloperoxidase foram avaliadas 5 h após o estímulo como marcadores de recrutamento de macrófragos e neutrófilos, respectivamente. O recrutamento celular total e diferencial na cavidade peritoneal foram avaliados 5 h após carragenina e 6 h após LPS. Parâmetros de estresse oxidativo foram avaliados na pata 3 h após o estímulo. A produção de citocinas e a ativação de NF-κB foram avaliadas 3 h após o estímulo, por ELISA. No modelo de inflamação aguda por LPS, o recrutamento de neutrófilos na cavidade peritoneal foi avaliado por imunofluorescência utilizando-se camundongos Lysm-eGFP. Adicionalmente, a produção de citocinas e a atividade de NF-κB foram avaliadas em macrófagos RAW 264.7 estimulados por LPS. Probucol, na dose de 3 mg/kg, inibiu dor manifesta, hiperalgesia mecânica e térmica; recrutamento celular na pata e na cavidade peritoneal; produção de citocinas e ativação de NF-κB em ambos os modelos. Nessa dose, não foi observado efeito antioxidante. Probucol, na concentração de 5 μM, inibiu produção de citocinas e atividade de NF-κB *in vitro.* Esses resultados contribuem para o estudo dos mecanismos anti-inflamatórios de probucol e demonstram seu potencial analgésico em modelos de inflamação aguda. Posteriormente avaliamos sua atividade analgésica em regime de pós-tratamento, contínuo, em modelo de dor persistente por CFA. Probucol, na dose de 3 mg/kg, inibiu hiperalgesia térmica e mecânica e recrutamento de neutrófilos na pata. Esses resultados foram acompanhados de inibição de TNF-α, IL-6 e IL-1β na pata e medula espinal. Conclui-se, portanto, que probucol apresenta efeito benéfico em modelos de inflamação aguda e sub-aguda a partir da inibição de NF-κB culminando na redução de produção de citocinas pró-hiperalgésicas e recrutamento celular.

Palavras-chave: Probucol. Dor. Inflamação. Citocinas. NF-κB.

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ABSTRACT

Probucol is a synthetic polyphenolic compound clinically used in the prevention and treatment of cardiovascular diseases. Nonetheless its beneficial effects have been reported in experimental models of other inflammatory diseases such as diabetes, Huntington's disease and ischemic injury. Probucol-mediated increase of endogenous antioxidants and inhibition of pro-inflammatory cytokines are among its putative mechanisms of actions. *In vitro* studies have demonstrated that probucol inhibits NF-κB activation and expression of adhesion molecules involved in cellular recruitment. Activation of NF-κB classical pathway is crucial for the production of pro-hyperalgesic mediators such as TNF-α, IL-6 and IL-1β in the context of inflammatory pain. In order to investigate the analgesic and anti-inflammatory potential of probucol, the present work used models of acute inflammation induced by carrageenan or lipopolysaccharide (LPS) and a model of subacute inflammation by complete Freund adjuvant (CFA). Overt pain-like behavior was evaluated by number of abdominal writhings during 20 minutes (induced by acetic acid or phenyl-p-benzoquinone) and number of paw flinches and time spent licking the paw over 30 minutes post CFA. In models of acute inflammation by carrageenan or LPS, mice received probucol p.o. 1 h before stimulus. Mechanical and thermal hyperalgesia were evaluated 1, 3 and 5 h post stimulus using a digital analgesiometer and hot plate, respectively. N-acetylglucosamine and myeloperoxidase activities were evaluated 5 h post stimulus as markers of macrophage and neutrophil recruitment, respectively. Total and differential cellular recruitment in peritoneal cavity were evaluated 5 h post carrageenan and 6 h post LPS. Parameters of oxidative stress were assessed in paw tissue 3 h post stimulus. Cytokine production and NF-κB activation were also evaluated 3 h post stimulus by ELISA. In the model of acute inflammation by LPS, neutrophil recruitment was also evaluated by immunofluorescence using Lysm-eGFP mice. Additionally, cytokine production and NF-κB activity were evaluated in RAW 264.7 macrophages stimulated with LPS. Probucol at 3 mg/kg inhibited overt pain-like behavior; mechanical and thermal hyperalgesia; cellular recruitment in paw and peritoneal cavity; production of cytokines as well as NF-κB in both models. At this dose we have not observed antioxidant effect. Probucol at 5 μM inhibited cytokine production and NF-κB activity *in vitro*. These results contribute to the elucidate probucol's anti-inflammatory mechanism and demonstrated its inflammatory potential in models of acute inflammation. Later we have evaluated its analgesic activity in post-treatment regimen in model of persistent pain induced by CFA. Probucol at 3 mg/kg inhibited mechanical and thermal hyperalgesia as well as neutrophil recruitment in paw. These results were accompanied by inhibition of TNF-α, IL-6 and IL-1β in paw and spinal cord. In conclusion we have shown that probucol presents beneficial effects in models of acute and subacute inflammation through inhibition of NF-κB. In turn it culminates in reduction of pro-hyperalgesia cytokines and cellular recruitment.

Key Words: Probucol. Pain. Inflammation. Cytokines. NF-κB.

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LISTA DE ABREVIATURAS E SIGLAS

ABCA1	ATP-binding cassette transporter A1
CFA	Adjuvante completo de Freund
DAMP	Padrão molecular associado a dano
ET-1	Endotelina 1
GSH	Glutationa reduzida
HDL	Lipoproteína de alta densidade
IL	Interleucina
IL-1R	Receptor de Interleucina 1
LDL	Lipoproteína de baixa densidade
LPS	Lipopolissacarídeo
MDA	Malondialdeído
MMP-1	Metaloproteinase 1
MMP-9	Metaloproteinase 9
NF-κB	Fator de transcrição nuclear kappa B
NOD	Receptor do tipo NOD
oxLDL	Lipoproteína de baixa densidade oxidada
PAMP	Padrão molecular associado a patógeno
PGE_2	Prostaglandina E2
PMA	Phorbol-12-miristato-13-acetato
PRR	Receptor de reconhecimento padrão
TNF-α	Fator de necrose tumoral α
TNFR	Receptor do fator de necrose tumoral
TOLL	Receptor do tipo toll
TRP	Receptor de potencial transitório
TRPM8	Receptor de potencial transitório subfamília m, membro 8
TRPV1	Receptor de Potencial Transitório Subfamília V, Membro 1
TTX-R	Canal tetrodoxina-resistente

SUMÁRIO

1 INTRODUÇÃO

1.1 Inflamação Aguda e Via Clássica do Fator de Transcrição NF-κB

O processo inflamatório agudo, em linhas gerais, pode ser definido como uma resposta coordenada por células do sistema imunológico frente ao dano tecidual ou a estímulos potencialmente nocivos. Tais estímulos podem ser de natureza química, como alterações de pH, ou física, como temperatura e pressão. Adicionalmente, uma ampla variedade de moléculas atua como ligante de receptores expressos na superfície de células do sistema imunológico, culminando em sua ativação e produção de mediadores inflamatórios (MEDZHITOV, 2008). Essas moléculas podem ser classificadas em dois grupos principais: padrões moleculares associados a patógenos (PAMPs) ou ao dano (DAMPs). Embora PAMPs sejam provenientes de micro-organismos e DAMPs sejam liberados pelo próprio hospedeiro, ambos atuam como ligantes de diversas classes de receptores de reconhecimento padrão (PRRs), como os receptores do tipo Toll (TLRs) e do tipo NOD (NLRs) (KONO; ROCK, 2008; MEDZHITOV, 2001).

O reconhecimento desses padrões moleculares por células residentes, como macrófagos e mastócitos, leva à ativação de cascatas de sinalização intracelulares culminando na produção e liberação de mediadores pró-inflamatórios. Tais mediadores agem de forma autócrina ou parácrina, amplificando o processo inflamatório. Dentre as vias de sinalização ativadas por PRRs, destaca-se a ativação clássica do fator de transcrição nuclear kappa B (NF-κB). NF-κB se refere a um grupo de dímeros, sendo o heterodímero p50-65 o de maior relevância na resposta inflamatória aguda. Sua ativação é crucial para a maioria dos eventos inflamatórios agudos, desde a ativação endotelial à sobrevivência dos leucócitos no micro-ambiente inflamatório (GHOSH; HAYDEN, 2008; WALMSLEY et al., 2005). Na ausência de estímulos, NF-κB permanece no citoplasma ligado às proteínas regulatórias IκB (IκBα, IκBβ e IκBε) (SCHEIDEREIT, 2006). A ativação de PRRs, de receptor de IL-1 (IL-1R) ou de receptor de TNF (TNFR) leva à ativação do complexo IKK, o qual é composto pelas

quinases IKKα e β e pela proteína regulatória IKKγ. O complexo IKK fosforila as proteínas inibitórias IκB sinalizando para sua ubiquitinação e degradação via proteassomo. Uma vez livre do complexo inibitório, a subunidade p65 do NF-κB é fosforilada e o fator de transcrição é então translocado para o núcleo, onde interage com o DNA promovendo a transcrição de genes pró-inflamatórios, como citocinas, quimiocinas e seus receptores e moléculas de adesão (GHOSH; HAYDEN, 2008). Esses mediadores, por sua vez, são fundamentais para as etapas de migração celular e posterior ativação das células recrutadas. Nesse contexto, IL-1β e TNF-α são liberados por células residentes ativadas por PAMPs ou DAMPs (com subsequente ativação da via do NF-κB) e se ligam a seus respectivos receptores na superfície de células endoteliais levando à secreção de quimiocinas e expressão de moléculas de adesão como E-selectinas e ICAM-1 (WYBLE et al., 1997). A ativação endotelial propicia o recrutamento de leucócitos, sobretudo neutrófilos e monócitos, para o foco da infecção ou injúria tecidual. No ambiente micro-inflamatório, IFN-γ e IL-6 contribuem para a diferenciação de monócitos em macrófagos (CHOMARAT et al., 2000; MUNDER et al., 1998). Adicionalmente, IL-6 favorece o recrutamento de monócitos sobre neutrófilos aumentando a secreção de quimioatraentes de monócitos e diminuindo de neutrófilos em células endoteliais (HURST et al., 2001; KAPLANSKI et al., 2003).

A modulação da via do NF-κB é de suma importância uma vez que seus próprios genes alvo podem regular positivamente a via prolongando a resposta inflamatória. Por exemplo, o lipopolissacarídeo (LPS), um produto bacteriano, ativa NF-κB via TLR4 e prolonga sua ativação pela indução de TNF-α e IL-1β (SEN; SMALE, 2010). Falhas na regulação da via estão intimamente associadas ao desenvolvimento de doenças inflamatórias crônicas (BALDWIN, 2001; HUGOT et al., 2001; SCHOTTELIUS; BALDWIN JR., 1999). Dessa forma, não é surpreendente a existência de múltiplos mecanismos endógenos modulando a ativação e atividade do fator de transcrição NF-κB. Tais mecanismos não só promovem a inibição da via como também garantem que a transcrição dos genes alvo siga um perfil temporal. Alguns promotores não requerem o remodelamento da cromatina para serem expostos ao NF-κB, sendo, pois, transcritos primeiramente. Além disso, a afinidade do fator de transcrição com os sítios de ligação ao DNA é variável, tornando alguns genes mais vulneráveis à inibição

de proteínas IκB do que outros (SEN; SMALE, 2010). Por meio desses mecanismos a via clássica do NF-κB é orquestrada a fim de que genes pró-inflamatórios sejam transcritos em um primeiro momento. Em contrapartida, genes anti-inflamatórios são transcritos mais tardiamente, controlando resposta inflamatória e seus efeitos deletérios sobre o tecido. Cao et al. (2006) demonstraram essa cinética em macrófagos RAW 264.7 estimulados com LPS. NF-κB ativa os promotores dos genes da citocina pró-inflamatória TNF-α e da anti-inflamatória IL-10 em tempos diferentes e os regula através de subunidades distintas. O promotor de TNF-α é regulado pela subunidade p65 e o pico da atividade se dá 6 horas pós-LPS enquanto o promotor de IL-10 é modulado por p50 com atividade 16 horas após estimulação com o agonista de TLR4 (CAO et al., 2006).

Em virtude da ampla variedade de mecanismos regulatórios, por vezes redundantes, a resposta inflamatória é cada vez mais vista como uma sequência de eventos altamente organizada. O perfil inicial de mediadores é claramente pró-inflamatório com liberação de IL-1β, TNF-α, IFN-γ, CXCL1. No entanto, as mesmas vias de sinalização, como NF-κB, promovem a transcrição de genes com atividades antagônicas ao primeiro grupo, regulando negativamente essas cascatas intracelulares iniciais. Os neutrófilos, primeiros leucócitos recrutados, são gradativamente substituídos por macrófagos devido ao *shift* de quimiocinas promovido por IL-6 e por entrarem em apoptose no micro-ambiente inflamatório (KAPLANSKI et al., 2003; SIMON, 2003). De forma sinérgica, esses eventos promovem a transição entre inflamação aguda e resolução, sendo esta um processo ativo de restauração da homeostase do tecido.

1.2 Dor Inflamatória

A dor, ou nocicepção, um dos cinco sinais cardinais da inflamação, é um dos motivos primários pelos quais a população procura atendimento médico. A dor inflamatória, ou dor aguda, é resultado da ativação de vias de sinalização evolucionariamente conservadas em resposta a estímulos nocivos, potencialmente danosos. É, certamente, um mecanismo de autopreservação, orquestrado por células

do sistema imunológico e do sistema nervoso (JI; CHAMESSIAN; ZHANG, 2016). A persistência da resposta inflamatória, portanto, não só leva ao dano tecidual e à perda de função, como também contribui para a cronificação da dor. A dor crônica, por sua vez, é incapacitante e acomete até 30% de adultos no mundo (JI; CHAMESSIAN; ZHANG, 2016). Sendo assim, além de caracterizar um problema de saúde pública, a alta prevalência de dor crônica apresenta desdobramentos sócio-econômicos por afetar diretamente a população economicamente ativa. O estudo dos mecanismos de nocicepção é fundamental para o desenvolvimento de terapias mais eficientes, tanto no tratamento da dor crônica, quanto na sua prevenção, uma vez que a cronificação está intimamente relacionada ao processo inflamatório persistente e não resolvido (JI; XU; GAO, 2014).

A percepção dolorosa se inicia pela ativação de neurônios sensoriais primários, que são componentes o sistema nervoso periférico. Seus corpos celulares se localizam no gânglio da raiz dorsal ou no gânglio trigeminal. Os neurônios do gânglio trigeminal projetam seus axônios para a cabeça e o da raiz da dorsal inervam o restante do corpo, como pele, músculo e articulações (BASBAUM et al., 2009; GOLD; GEBHART, 2010; JI; XU; GAO, 2014). Os neurônios sensoriais primários são divididos em três classes principais conforme seu diâmetro, presença de bainha de mielina e tipo de estímulo que os ativam. As fibras Aα e Aβ são mielinizadas, de maior diâmetro e estão envolvidas na propriocepção, tato e estímulos inócuos em geral. As fibras Aδ e C, em contrapartida, participam da transdução de estímulos dolorosos. As fibras Aδ apresentam diâmetro médio, são mielinizadas, apresentando, portanto, condução rápida. Podem ser subdivididas em tipo I e II por apresentarem limiar térmico distintos, além de serem ativadas por diferentes estímulos. As fibras C, por sua vez, apresentam diâmetro menor e condução lenta (não são mielinizadas). Ambas as classes, fibras Aδ e C, reconhecem estímulos nociceptivos de natureza química, térmica e mecânica (JULIUS; BASBAUM, 2001).

Tais estímulos são reconhecidos por receptores ou canais iônicos expressos na superfície dos nociceptores levando à transdução, fenômeno pelo qual esses estímulos são transformados em atividade elétrica. Em linhas gerais, a interação ligante – receptor leva à abertura de canais iônicos com subsequente alteração da

permeabilidade iônica e despolarização periférica (BRAZ et al., 2014). O potencial de ação é, então, conduzido dos axônios para os corpos celulares organizados nos gânglios da raiz dorsal ou trigeminal e transmitido para outros neurônios nociceptivos. No sistema nervoso central, o estímulo é interpretado no córtex somatossensorial (TODD, 2010).

Dentre os canais iônicos que participam da transdução e reconhecimento de sinais danosos, pode-se destacar alguns canais da família TRP (Receptor de Potencial Transitório). O canal TRPV1 (Receptor de Potencial Transitório Subfamília V, Membro 1) é sensível a calor, pH ácido e apresenta também como ligante a capsaicina (O'NEILL et al., 2012). Já o canal TRPM8 (Receptor de Potencial Transitório Subfamília M, Membro 8) é sensível a baixas temperaturas e a estímulos químicos como mentol e óleo de mostarda (JANSSENS et al., 2016). Além dos membros da família TRP, outros canais iônicos estão envolvido na transdução de estímulos nocivos. Os canais de sódio tetrodoxina-resistentes (TTX-R), como $Na_v1.8$, são expressos em neurônios do gânglio da raiz dorsal e sua inibição melhora dor inflamatória por carragenina e hiperalgesia térmica induzida por capsaicina (AKOPIAN et al., 1999; AKOPIAN; SIVILOTTI; WOOD, 1996; LAIRD et al., 2002).

Além de estímulos físicos e térmicos, nociceptores respondem a mediadores inflamatórios, PAMPs e DAMPs através de receptores em sua superfície. No contexto de dor inflamatória, sobretudo, os mediadores pró-inflamatórios são cruciais para o desenvolvimento de hiperalgesia mecânica e térmica (CALIL et al., 2014; CUNHA et al., 2005, 2008). Agonistas de TLR4, como LPS e carragenina, ativam a via clássica do NF-κB levando à secreção sequencial de citocinas pró-hiperalgésicas. TNF-α é o primeiro mediador detectado, seguido de CXCL1, IL-1β e aminas simpáticas. Alternativamente, TNF-α promove a liberação de IL-6 seguida de IL-1β (CALIL et al., 2014; CUNHA et al., 2005). IL-1β por sua vez age diretamente sobre neutrófilos estimulando a produção de prostaglandina E_2 (PGE_2). Esse mediador lipídico contribui para a resposta hiperalgésica, sensibilizando neurônios nociceptivos (CUNHA et al., 2008). Alternativamente, estudos prévios já demonstraram a expressão de receptores de IL-1β e TNF-α em células neuronais, sugerindo a atuação direta desses mediadores sobre os neurônios (BINSHTOK; WANG; ZIMMERMANN, 2008; HUDMON et al., 2008;

JIN; GEREAU IV, 2006). Jin et al. (2006) demonstraram que TNF-α se liga a TNFR1 em neurônios do gânglio da raiz dorsal levando à ativação da quinase p38. Esta, por sua vez, fosforila o canal de sódio $Na_v1.8$ provocando despolarização do nociceptor (JIN; GEREAU IV, 2006). O papel de IL-6 em mecanismos de hiperalgesia ainda não está bem elucidado na literatura. Seu aumento foi detectado na medula espinal de ratos em modelo de inflamação sub-aguda por CFA, sugerindo sua participação em vias de nocicepção (SUN et al., 2012; XU et al., 2014). Vazquez et al. (2012) demonstraram amplificação da resposta inflamatória e hiperalgésica após administração intra-articular de IL-6. Em concordância, a administração intratecal de gp130 solúvel (inibidor da interação IL-6 – receptor) diminuiu a sensibilização central, ratificando o papel pró-hiperalgésico de IL-6 (VAZQUEZ et al., 2012).

A dor inflamatória é, portanto, um clássico exemplo de interação neuro-imune, isto é, de modulação recíproca entre células do sistema nervoso central, sobretudo nociceptores, e células do sistema imunológico. Assim, seu controle está intimamente associado ao controle da resposta inflamatória em si (JI; CHAMESSIAN; ZHANG, 2016). A persistência do estímulo nocivo ou o comprometimento dos mecanismos de resolução são importantes fatores para a manutenção da resposta inflamatória, contribuindo para o dano tecidual e cronificação da dor (JI; XU; GAO, 2014). A dor, quando patológica, perde seu caráter de auto-preservação, prejudicando a qualidade de vida dos pacientes.

1.3 Probucol

Probucol ([4,4′-isopropylidine dithio]-bis[2,6-di-t-butyl-phenol]) é um composto polifenólico utilizado na prevenção e tratamento de doenças cardiovasculares. Probucol age diretamente sobre o metabolismo lipídico, acelerando a degradação de LDL e inibindo a bio-síntese e absorção de colesterol (YAMAMOTO, 2008). No entanto, estudos em humanos observaram redução também nos níveis séricos de HDL inviabilizando sua comercialização sob a ótica dos órgãos de saúde de muitos países ocidentais (BUCKLEY et al., 1989; YAMAMOTO, 2008). No Brasil, foi

comercializado sob o nome fantasia Lesterol® (Sanofi Aventis, São Paulo, Brasil) até 2004. Um dos mecanismos propostos para esse efeito adverso seria a inibição de probucol sobre a proteína transportadora de colesterol das células para apolipoproteínas séricas ABCA1 (FAVARI et al., 2004). Nesse estudo, Favari et al. (2004) observaram a maior retenção de colesterol em macrófagos J774 tratados com probucol. Em contrapartida, Yamamoto et al. (1988) demonstraram menor incidência de formação de células espumosas na presença de probucol. Os resultados foram atribuídos à diminuição no acúmulo de lipídeos e aumento de efluxo de colesterol em células THP-1 (linhagem monocítica humana) e, principalmente, à inibição da oxidação de LDL (PARTHASARATHY et al., 1986; YAMAMOTO et al., 1988). A internalização de LDL acetilado, produto da modificação oxidativa de LDL, por macrófagos é um importante evento associado à formação de células espumosas e aterogênese (WOOLLARD; GEISSMANN, 2010). A partir desses resultados, sugere-se então que probucol apresente potencial terapêutico mais amplo, e não somente efeitos sobre o metabolismo lipídico.

Nesse contexto, os efeitos antioxidantes de probucol foram investigados. Dados na literatura demonstram que probucol apresenta ação antioxidante por atuar como *scavenger* de ânion superóxido *in vitro* (BRIDGES; SCOTT; BELCH, 1991). Em modelos animais, probucol aumenta níveis de glutationa reduzida (GSH) e atividade de enzimas antioxidantes endógenas como glutationa peroxidase e superóxido dismutase (AL-MAJED, 2011; COLLE et al., 2013; SIVESKI-ILISKOVIC; KAUL; SINGAL, 1994; ZHANG et al., 2009). Adicionalmente, probucol reduz parâmetros de lipoperoxidação, diminuindo níveis de malondialdeído (MDA) (AL-MAJED, 2011). Suas propriedades antioxidantes são atribuídas aos grupamentos –OH em sua estrutura (Figura 1).

Em relação a suas propriedades anti-inflamatórias, vários estudos demonstraram que probucol inibe a produção de citocinas pró-inflamatórias *in vitro* e *in vivo*. Em modelos experimentais de diabetes mellitus tipo 2 e aterosclerose, probucol diminuiu os níveis séricos de TNF-α e IL-6 (LI et al., 2011; ZHANG et al., 2009). Adicionalmente, a inibição sobre IL-1β foi descrita *in vitro* por diversos autores (AKESON et al., 1991; KU et al., 1988; LIU et al., 2000; MENG et al., 2004). Ozaki et al.

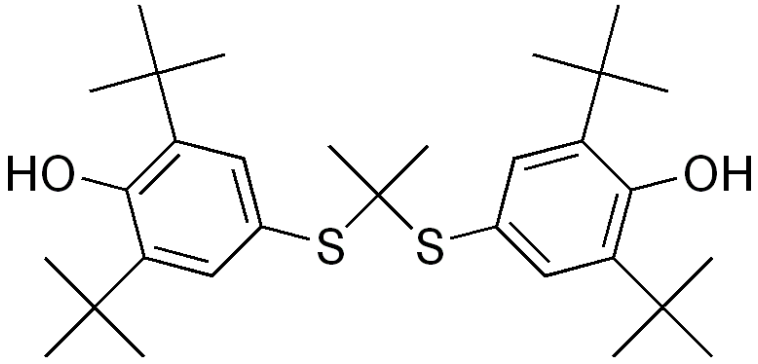
(1999) demonstraram redução na produção de PGE2 por células mesangiais pré-tratadas com probucol e estimuladas com LDL oxidado (oxLDL) (OZAKI et al., 1999). Tais mediadores participam de vários eventos da resposta inflamatória promovendo recrutamento e ativação celular, além de atuarem diretamente na sinalização nociceptiva. Sua transcrição e subsequente produção estão diretamente relacionadas à ativação clássica do fator de transcrição NF-κB, seja por PRRs ou receptores das citocinas IL-1β ou TNF-α. Assim sendo, um possível mecanismo anti-inflamatório de probucol seria a inibição de NF-κB. De fato, probucol inibiu a translocação do fator de transcrição para o núcleo em cultura de células endoteliais estimuladas com IL-1β, TNF-α, angiotensina II ou em hipóxia (AOKI et al., 2001; CHANG et al., 2010; CHEN et al., 2003; ZHANG et al., 2013). Em contrapartida, sob nosso conhecimento, o efeito inibitório de probucol sobre a ativação ou atividade de NF-κB ainda não foi demonstrado *in vivo* na literatura. Fu et al. (2015) observaram que tratamento contínuo com probucol (8 semanas) diminuiu a expressão total de NF-κB no átrio esquerdo de coelhos submetidos ao modelo experimental de diabetes por aloxana (FU et al., 2015). Vale ressaltar que, embora esse dado contribua para a hipótese de que o fator de transcrição seja um alvo de probucol, o estudo não avalia ativação ou atividade do fator de transcrição NF-κB.

Além de reduzir níveis de citocinas e mediadores lipídicos pró-inflamatórios e hiperalgésicos, probucol inibe também a expressão de moléculas de adesão. Em células endoteliais ativadas por IL-1β ou TNF-α, probucol inibe a expressão de VCAM-1, ICAM-1 e E-selectina de maneira dose-dependente (FERNS et al., 1993; KANEKO et al., 1996; ZAPOLSKA-DOWNAR et al., 2000). Em menores concentrações, 5 μM, probucol reduz apenas expressão de VCAM-1 (CHEN et al., 2003). Probucol na concentração de 50 μM, em contrapartida, inibiu a expressão de VCAM-1, ICAM-1 e E-selectina (KANEKO et al., 1996; ZAPOLSKA-DOWNAR et al., 2000). Em concordância com esses resultados, em modelos experimentais de aterosclerose, probucol reduziu a infiltração de macrófagos em placas de ateroma, bem como a expressão de metaloproteinases MMP-1 e MMP-9 (LI et al., 2014, 2011). Tais efeitos contribuem diretamente para o aumento da estabilidade da placa, elucidando melhor o efeito anti-aterogênico de probucol. Adicionalmente, vale ressaltar que os mediadores discutidos

até então – citocinas pró-inflamatórias e hiperalgésicas, moléculas de adesão, metaloproteinases – são produzidos e secretados de maneira dependente de NF-κB, ratificando a hipótese de que esse fator de transcrição seja um alvo *upstream* de probucol.

Os mecanismos anti-inflamatórios e antioxidantes de probucol demonstrados até o momento elucidam, portanto, seu efeito benéfico em modelos experimentais de outras doenças (Tabela 1). Seu potencial terapêutico foi observado em modelos animais de doenças neurodegenativas, como Huntington (COLLE et al., 2013); metabólicas, como diabetes (FU et al., 2015; ZANARDO et al., 2003; ZHANG et al., 2009); de natureza isquêmica (AL-MAJED, 2011; PARK et al., 2007); e aterosclerose (LI et al., 2014, 2011; NIIMI et al., 2013). Considerando que tais doenças sejam de cunho inflamatório, buscamos avaliar, no presente trabalho, o efeito de probucol em três modelos experimentais de dor inflamatória: (a) modelo de dor inflamatória aguda induzida por carragenina; (b) modelo de dor inflamatória aguda induzida por lipopolissacarídeo; (c) modelo de dor inflamatória sub-aguda induzida por adjuvante completo de Freund (CFA).

Figura 1 – Estrutura química de probucol



Fonte: NIH PubChem (2016)

Tabela 1 – Efeitos Anti-inflamatórios e Antioxidantes de probucol

Efeito	Modelo Experimental	Referências
Inibição de IL-1β	Cultura de macrófagos estimulados por PMA ou LPS	AKESON et al., 1991; LIU et al., 2000; MENG et al., 2004
Inibição de TNF-α	Diabetes induzida por dieta rica em gordura (ratos) e aterosclerose (coelhos)	LI et al., 2011; ZHANG et al., 2009
Inibição de IL-6	Diabetes induzida por dieta rica em gordura (ratos) e aterosclerose (coelhos)	LI et al., 2011; ZHANG et al., 2009
Inibição de moléculas de adesão	Céluas endoteliais estimuladas por IL-1β ou TNF-α	FERNS et al., 1993; KANEKO et al., 1996; ZAPOLSKA-DOWNAR et al., 2000
Inibição da produção de PGE_2	Células mesangiais estimuladas com LDL oxidado	OZAKI et al., 1999
Inibição da ativação de NF-κB	Células endoteliais estimuladas com IL-1β, TNF-α, angiotensina II ou em hipóxia	AOKI et al., 2001; CHANG et al., 2010; CHEN et al., 2003; ZHANG et al., 2013
Inibição do recrutamento celular	Aterosclerose (coelhos)	LI et al., 2014, 2011
Aumento dos níveis de antioxidantes endógenos	Isquemia cerebral (ratos), doença de Huntington (ratos), diabetes induzida por dieta rica em gordura (ratos), cardiomiopatia induzida por adriamicina (ratos)	AL-MAJED, 2011; COLLE et al., 2013; ZHANG et al., 2009; SIVESKI-ILISKOVIC; KAUL; SINGAL, 1994
Diminuição de parâmetros de lipoperoxidação	Isquemia cerebral (ratos)	AL-MAJED, 2011

Fonte: o próprio autor

2 OBJETIVOS

2.1 Objetivo Geral

Avaliar o efeito de probucol em modelos de dor inflamatória aguda por carragenina ou por lipopolissacarídeo e em modelo de dor inflamatória sub-aguda por adjuvante completo de Freund.

2.2 Objetivos Específicos

Como objetivos específicos teve-se:

- Realizar curva de dose-resposta de probucol utilizando como parâmetros: dor manifesta, hiperalgesia mecânica e térmica, edema ou recrutamento leucocitário;
- Avaliar o efeito de probucol sobre recrutamento leucocitário no tecido subplantar e na cavidade peritoneal;
- Avaliar o efeito de probucol sobre a produção de citocinas pró-inflamatórias e hiperalgésicas;
- Avaliar o efeito de probucol sobre o estresse oxidativo e a capacidade antioxidante do tecido;
- Avaliar o efeito de probucol sobre a ativação e / ou atividade do fator de transcrição NF-κB.

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3 ARTIGO SUBMETIDO (INFLAMMATION RESEARCH)

O presente trabalho foi realizado na Universidade Estadual de Londrina, no Laboratório de Dor, Inflamação, Neuropatia e Câncer e submetido à revista Inflammation Research sob o título “Probucol attenuates overt pain-like behavior and carrageenan-induced inflammatory hyperalgesia and leukocyte recruitment by inhibiting NF-κB activation and cytokine production without antioxidant effects”.

Probucol attenuates overt pain-like behavior and carrageenan-induced inflammatory hyperalgesia and leukocyte recruitment by inhibiting NF-κB activation and cytokine production without antioxidant effects.

Amanda Z. Zucoloto[1], Marília F. Manchope[1], Larrisa Staurengo-Ferrari[1]; José C. Alves-Filho[2], Thiago M. Cunha[2], Maísa M. Antunes[3], Gustavo B. Menezes[3], Fernando Q. Cunha[2], Rubia Casagrande[4], Waldiceu A. Verri, Jr.[1]

Keywords: Probucol, Carrageenan, Pain, Acute Inflammation.

[1] Departamento de Ciências Patológicas, Centro de Ciências Biológicas, Universidade Estadual de Londrina, Rod. Celso Garcia Cid PR445 km 480, CEP 86057-970, Londrina, Paraná Caixa Postal 10.011, Brasil

[2] Department of Pharmacology, Ribeirão Preto Medical School, University of São Paulo, Av. Bandeirantes 3900, CEP 14049-900, Ribeirão Preto, São Paulo, Brasil

[3] Departamento de Morfologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Avenida Presidente Antonio Carlos 6627, CEP 31270-901 Belo Horizonte, Minas Gerais, Brasil

[4] Departamento de Ciências Farmacêuticas, Centro de Ciências da Saúde, Universidade Estadual de Londrina, Av. Robert Koch, 60, CEP 86038-350, Londrina, Paraná, Brasil

Correspondence: Waldiceu A. Verri Jr.; waldiceujr@yahoo.com.br; waverri@uel.br; + 55 43 3371 4979

ABSTRACT

Objective and design: This study aimed to evaluate the effect of probucol in inflammatory hyperalgesia and leukocyte recruitment in mice.

Treatment: Probucol at 0.3-3 mg/kg was administrated per oral 1 h before inflammatory stimulus.

Methods: Overt pain-like behaviors were determined by the number of abdominal writhings induced by phenyl-p-benzoquinone and acetic acid. Mechanical and thermal hyperalgesia induced by carrageenan were determined using an electronic anesthesiometer and hot plate apparatus, respectively. Leukocyte recruitment was evaluated by direct count or by determination of myeloperoxidase and *N*-acetylglucosaminidase activities. Antioxidant ability was determined by measurement of GSH levels, ABTS and FRAP assays. Cytokine production and NF-κB activation were evaluated by ELISA. Data were analyzed by ANOVA followed by Tukey's post-hoc. $p<0.05$ was considered significant.

Results: Probucol reduced overt pain-like behavior, and carrageenan-induced mechanical and thermal hyperalgesia. These effects were accompanied by reduced leukocyte influx in both paw skin and peritoneum exudate. Probucol did not alter carrageenan-induced tissue antioxidant capacity at anti-inflammatory/ analgesic dose. On the other hand, probucol inhibited carrageenan-induced IL-1β, TNF-α and CXCL1 production as well as NF-κB activation.

Conclusion: Probucol presents analgesic and anti-inflammatory activities by employing mechanisms other than its antioxidant properties. These mechanisms involve targeting of pro-inflammatory cytokines and NF-κB activation.

INTRODUCTION

Inflammation is a physiological response to noxious stimuli and tissue damage. It is often described as a cascade of events coordinated by immune cells in an effort to reestablish tissue homeostasis [1]. Nonetheless, the role of nociceptive neurons in inflammatory processes has been a subject of extensive research as inflammation-derived products were shown to activate nociceptive neurons [2–5]. In fact, pain, which is one of the hallmarks of inflammation, is an outcome of neuroimmune interactions. Cytokines, chemokines and lipid mediators secreted by immune cells can directly sensitize nociceptors leading to increased responsiveness and hyperalgesia. For example, IL-1β and tumor necrosis factor (TNF)-α signal through membrane receptors in sensory neurons leading to kinase-dependent modulation of ion channels, such as tetrodotoxin (TTX)-resistant sodium channels [3,4,6]. These events depend on posttranslational modifications; therefore they rapidly increase the responsiveness of nociceptors contributing to acute inflammatory pain. Cytokines can also act indirectly by inducing the production of hyperalgesic molecules capable of activating or sensitizing nociceptive neurons such as prostaglandin E_2 [7].

Production and secretion of proinflammatory mediators are triggered upon detection of pathogens through pattern recognition receptors (PRRs) or products released by necrotic cells such as ATP and IL-33 [1,5,8,9]. Activation of resident inflammatory cells through a wide range of PRRs and damage-associated molecular pattern (DAMP) receptors leads to activation of nuclear factor kappa B (NF-κB) [10,11]. NF-κB translocation to nucleus initiates the transcription of pro-inflammatory cytokines (IL-1β, TNF-α, IL-6, IL-8) and adhesion molecules (E-selectin, ICAM-1, VCAM-1) [12]. Furthermore, oxidative stress arises in the inflammatory microenvironment due to tissue injury, depletion of antioxidant molecules and increased production of reactive oxygen species (ROS) [11]. ROS contribute to both neutrophil recruitment and pain [13,14]. The net result is further amplification of the inflammatory response and its deleterious effects.

Inflammation and pain underlie nearly every pathophysiological process and are primarily treated with non-steroidal anti-inflammatory drugs (NSAIDs). The side effects associated with long-term use of NSAIDs and steroids stimulate the development of novel anti-inflammatory therapies [15,16]. Natural and synthetic products have been extensively exploited as sources of new drugs. For instance, naturally occurring polyphenolic compounds present analgesic activity via antioxidant and anti-inflammatory mechanisms in experimental models of inflammatory pain [17–19]. In turn, synthetic compounds with antioxidant properties have also been evaluated for putative analgesic effect [20–22].

Probucol is a synthetic molecule clinically used for prevention and treatment of hypercholesterolemia and atherosclerosis. In contrast to other lipid-lowering drugs, probucol presents antioxidant properties by acting as free radical scavenger and by increasing endogenous antioxidants [23–26]. Regarding its anti-inflammatory activity, probucol was shown to inhibit secretion of pro-inflammatory cytokines and expression of adhesion molecules [27–32]. The beneficial use of probucol has been evaluated under various disease models where inflammation plays a pivotal role such as diabetes [27,33,34], Huntington's disease [26], brain ischemia [25,35] and cardiovascular disorders [24,29,36–38]. Considering that most of these findings are from *in vitro* studies and experimental models of chronic diseases, the present study aimed to evaluate the effect of probucol in the context of acute inflammation and pain.

MATERIALS AND METHODS

Animals

Male Swiss mice (25-30g) from Londrina State University, Parana, Brazil were used in this study. Mice were housed in standard clear plastic cages, and received food and water *ad libitum* under a 12:12 h light/dark cycle at 21 °C. All behavior testing were performed between 9 am and 5 pm in temperature-controlled room. The Animal Welfare and Ethics Committee of Londrina State University approved this study (process number 1012.2015.74). All efforts were made to minimize the number of animals used and their suffering.

Drugs

Materials were obtained from the following sources: probucol (Santa Cruz Biotechnology, Dallas, TX, USA), naringenin (Santa Cruz Biotechnology, Dallas, TX, USA), acetic acid (Mallinckrodt Baker, S.A., Mexico City, Mexico), phenyl-p-benzoquinone (Sigma Chemical Company, St. Louis, MO, USA), and carrageenan (Santa Cruz Biotechnology, Dallas, TX, USA).

Experimental procedures

Mice were treated with probucol (0.3, 1, 3 mg/kg, per oral) or vehicle (Tween 80 5% in saline) 1 h before the administration of the inflammatory stimulus. Overt pain-like behavior was induced by i.p. injection of phenyl-p-benzoquinone (PBQ) (1890 μg/kg) or acetic acid 0.8% (10 mL/kg) and the writhing response was evaluated during 20 min. Paw edema, mechanical hyperalgesia and thermal hyperalgesia were evaluated 1, 3 and 5 h after i.pl. administration of carrageenan (300 μg/20 μL). Paw skin was removed for assessment of leukocyte recruitment by myeloperoxidase (MPO) and *N*-acetylglucosaminidase (NAG) activity assays 5 h after carrageenan injection. Leukocyte recruitment into the peritoneal cavity was assessed 5 h after i.p. administration of carrageenan (1 mg/200 μL/cavity). ABTS (2,2'-Azinobis-3-ethylbenzothiazoline 6-sulfonic acid), FRAP (ferric reducing antioxidant power) and GSH (reduced glutathione) assays were performed using paw skin collected 3 h after carrageenan injection. IL-1β, CXCL1, TNF-α, total NF-κB p65 subunit and phosphorylated NF-κB p65 subunit levels were evaluated 3 h after carrageenan injection in paw by ELISA. IL-1β, CXCL1 and TNF-α levels were evaluated in peritoneal exudates collected 5 h after carrageenan injection. Total NF-κB p65 subunit and phosphorylated NF-κB p65 subunit levels were evaluated in peritoneal adherent macrophages collected 3 h after carrageenan injection plus 4 h of adherence *in vitro*. Doses of stimuli and time points of sample collection were based on previous studies in our laboratory [39,40].

Overt pain-like behavioral tests

For evaluation of writhing response, PBQ (1890 μg/kg, DMSO 2%, v/v in saline) was administrated into the peritoneal cavity of mice pre-treated with probucol (0.3 – 3 mg/kg, p.o.). The intensity of overt pain-like behavior is expressed as the number of writhings over 20 min after stimulus injection. The dose of probucol of 0.3 mg/kg was chosen for the acetic acid-induced writhing model and writhing response was also observed for 20 min post stimulus injection [20,41].

Mechanical hyperalgesia test

Mechanical hyperalgesia was assessed by an electronic version of von Frey filaments [42]. A handheld force transducer (electronic anesthesiometer; Insight, Ribeirao Preto, SP, Brazil) adapted with a 0.5 mm^2 polypropylene tip was used to evoke hind paw nociceptive withdrawal response. The intensity of the pressure (in g) at the moment of paw withdrawal was automatically recorded. The mechanical threshold was tested before (baseline) and after carrageenan i.pl. administration (300 µg/paw). The results are expressed as delta (Δ) withdrawal threshold (in g), obtained by subtracting the measurements at each time point (1, 3 and 5 h after i.pl. carrageenan injection) from the baseline values.

Thermal hyperalgesia test

Animals were place on a hot plate apparatus (Insight, Ribeirao Preto, SP, Brazil) at a constant temperature of 52 °C. The end-point was characterized by removal of the paw followed by flinching or licking the paw, and latency time until the end-point reaction was determined. Maximum latency until end-point was set at 25 s to avoid tissue damage. The results are expressed as means of latency (s) within experimental groups.

Edema assessment

Paw edema was measured using a dial thickness gauge 0-20 mm (Mitutoyo, Andover, Hampshire, UK). The results are expressed as paw thickness (in mm). The values were obtained by subtracting the baseline values from the measurements obtained at each time point (1, 3 and 5 h after i.pl. carrageenan injection).

MPO and NAG activity assays

MPO and NAG kinetic-colorimetric assays were performed as indirect indicators of neutrophil and macrophage recruitment to paw tissue, respectively. Paw skin samples were collected 5 h after carrageenan injection and homogenized in ice-cold K_2HPO_4 buffer (400 µL, 50 mM, pH 6.0) containing HTAB (0.5% weight/volume) using a tissue-tearor (Biospec, Bartlesville, OK, USA). Samples were centrifuged (16100 *g* x 2 min x 4 °C) and the supernatants were used for both assays. The MPO activity assay was performed as previously described [43]. Briefly, 50 mM phosphate buffer, pH 6.0 containing 0.167 mg/ mL o-dianisidine dihydrochloride and 0.015 % hydrogen were added to samples and the absorbance was read at 450nm (Multiskan GO Microplate Spectrophotometer, Thermo Scientific, Vantaa, Finland). The values obtained were compared to a standard curve of neutrophils and results are expressed as MPO activity (neutrophils x 10^6 / g of tissue). The NAG activity assay was performed as previously described [44]. Samples were diluted in citrate/phosphate buffer (0.1 M citric acid, 0.1 M Na_2HPO_4, pH 4.5) in a proportion of 5:1. p-nitrophenyl-N-acetyl-β-glucosaminide dissolved in citrate/phosphate buffer was added to samples followed by addition of 0.2 M glycine buffer (pH 10.6). The absorbance was read at 405nm (Multiskan GO, Thermo Scientific). The values obtained were compared to a standard curve of macrophages and results are expressed as NAG activity (macrophages x 10^6 / g of tissue).

Leukocyte recruitment into the peritoneal cavity

Leukocyte recruitment into the peritoneal cavity was assessed 5 h after i.p. administration of carrageenan (1 mg/cavity). The dose of the inflammatory stimulus was based on a dose response assessment performed in our laboratory (data not shown). Peritoneal cavities were washed with 1 mL of phosphate-buffered saline (PBS). Total leukocyte counts were performed after dilution of peritoneal exudate in Turk solution (2% acetic acid) using a Neubauer chamber. Differential cell counts were performed using the Fast Panoptic Kit for histological analysis (Laborclin, Pinhas, BR, Brazil) to distinguish polymorphonuclear (PMNs) from mononuclear cells under a light microscope (Olympicus Optical Co., Hamburg, Germany). The results are expressed as number of cells x 10^6 per cavity.

ABTS and FRAP assays

ABTS and FRAP assays were used to evaluate the tissue antioxidant capacity. Both tests were adapted to a 96-well microplate format as previously described [22]. Paw skin samples were collected 3 h after i.pl. stimulus (carrageenan 300 µg/paw) and homogenized with a tissue-tearor in ice-cold KCl buffer (500 µL, 1.15% w/v). Samples were centrifuged at 835 *g* at 4 °C for 10 min, and supernatants were used in both assays. ABTS assay was performed as an indicator of the sample's ability to scavenge the free radical ABTS. Diluted ABTS solution was added to samples and the absorbance was measured at 730 nm after 6 min of incubation at 25 °C (Multiskan GO, Thermo Scientific). In turn, FRAP assay evaluates the ferric reducing ability of the samples. Freshly prepared FRAP reagent was added to samples and incubated for 30 min at 37 °C prior to absorbance measurement at 595 nm (Multiskan GO, Thermo Scientific). The results for both assays were equated against a standard Trolox curve (0.02-20 nmol) and expressed as nmol Trolox eq. per mg of tissue.

GSH levels measurement

Paw skin samples were collected 3 h after i.pl. stimulus (carrageenan 300 µg/paw) and stored at -80 °C for at least 48 hours. For the assay, samples were homogenized with a tissue-tearor with 200 µL of 0.02 M EDTA after thawing. Trichloroacetic acid 50% (TCA) was added to homogenates and the mixture was homogenized for 15 min using a vortex followed by centrifugation (1500 *g* x 15 min x 4 °C). The supernatants were added to a 96-well microplate followed by addition of 0.2 M TRIS buffer, pH 8.2, and 0.01 M DTNB. The absorbance was measured at 412 nm (Multiskan GO, Thermo Scientific) after 5 min incubation at room temperature. The values obtained were compared to a standard curve of GSH. The results are expressed as GSH levels (nmol) per mg of tissue [22].

Cytokine measurement

Paw skin samples were collected 3 h after stimulus (carrageenan 300 µg/paw). Samples were homogenized with a tissue-tearor in 500 µL of ice-cold buffer with protease inhibitors followed by centrifugation (835 *g* x 15 min x 4 °C). Peritoneal exudates were collected using a micropipette after washing the peritoneal cavity with 1mL of phosphate-buffered saline (PBS) 5 h after stimulus (carrageenan 1 mg/cavity.) The supernatants of paw skin homogenates and peritoneal exudates were used to determine IL-1β, TNF-α and CXCL1 levels by enzyme-linked immunosorbent assay (ELISA) using commercial kits (eBioscience, San Diego, CA, USA). Absorbance was

measured at 450 nm (Multiskan GO, Thermo Scientific). The results are expressed as picograms of cytokines per g of tissue or mL.

NF-κB activation

Paw skin samples were collected 3 h after stimulus (carrageenan 300 µg/paw). Samples were homogenized with a tissue-tearor in 400 µL of ice-cold lysis buffer (Cell Signaling, Danvers, MA, USA) followed by centrifugation (16100 *g* x 10 min x 4 °C). In another set of experiments, peritoneal exudates were harvested 3 h after stimulus (carrageenan 1 mg/cavity). Cells were counted using a Neubauer chambers and $2x10^5$ cells were plated on 24-well plates in RPMI medium and incubated at 37°C, 5% CO_2. Four hours later cells were washed with PBS to remove non adherent cells and 500 µL of ice-cold lysis buffer was added. Cells were sonicated and centrifuged (835 *g* x 10 min x 4 °C). The supernatants were used for determination of phosphorylated and total levels of NF-κB p65 subunit using ELISA PathScan Kits (Cell Signaling, Danvers, MA, USA) according to the manufacturer's directions. Absorbance was measured at 450 nm (Multiskan GO, Thermo Scientific). The results are expressed as IOD ratio (p- NF-κB p65/total NF-κB p65).

Statistical analyses

Results are presented as mean ± SEM of measurements made on 6 mice per group per experiment, and are representative of two independent experiments. Two-way repeated measures analysis of variance (ANOVA) followed by Tukey's post hoc were used to compare groups and doses at all time points when responses were measured at different time points after stimulus injection (mechanical and thermal hyperalgesia tests and edema assessment). One-way ANOVA followed by Tukey's post-hoc was performed for data from single time point experiments. $P < 0.05$ was considered statistically significant. All data analyses were performed using GraphPad Prism® 5.0 (GraphPad Software, Inc., USA-500.288), as well as elaboration of figures.

RESULTS

Probucol inhibits PBQ- and acetic acid-induced writhing responses

Mice were treated with probucol (0.3, 1, 3 mg/kg, p.o.) or vehicle (Tween 80 5% in saline) 1 h before i.p. administration of PBQ (1890 µg/kg). Probucol at doses of 0.3 mg/kg and 3 mg/kg reduced 75% and 73% of PBQ-induced writhing response, respectively (Fig. 1a). At 1 mg/kg, the inhibitory effect of probucol was not statistically different from the group that received vehicle only ($p > 0.05$). Probucol produced a bell-shaped dose-dependent analgesic effect in the PBQ model. The dose of 0.3 mg/kg of probucol was chosen for evaluation of its analgesic effect in acetic acid-induced overt pain-like behavior. Over a period of 20 min, pretreatment with probucol reduced 48% the number of writhings ($p < 0.05$) (Fig. 1b).

Probucol inhibits carrageenan-induced mechanical and thermal hyperalgesia

Mice were treated with probucol (0.3, 1, 3 mg/kg, p.o.) or vehicle (Tween 80 5% in saline) 1 h before i.pl. administration of carrageenan (300 µg/paw). Mechanical and thermal hyperalgesia were evaluated at 1, 3 and 5 h after carrageenan injection. Carrageenan induced mechanical and thermal hyperalgesia at all times points. Probucol

at doses of 0.3 and 1 mg/kg did not inhibit carrageenan-induced mechanical and thermal hyperalgesia. Probucol at 3 mg/kg in turn reduced mechanical hyperalgesia at all time points (up to 72%). The animals within this experimental group showed similar levels of intensity of mechanical hyperalgesia to saline group at 1 and 5 h ($p > 0.05$) (Fig. 2a). Three mg/kg of probucol inhibited carrageenan-induced thermal hyperalgesia at all time points, up to 86% at 3 h. Thermal threshold was not statistically different from saline group at any of the evaluated time points ($p > 0.05$) (Fig. 2b). In addition, the dose of 3 mg/kg showed a significant inhibition compared to the lower doses at most time points, especially at 3 h (peak of hyperalgesia), for both mechanical and thermal hyperalgesia tests ($p < 0.05$). The probucol inhibition of carrageenan-induced mechanical hyperalgesia was also in a bell-shape (Fig. 2a) as for PBQ model (Fig. 1a).

Probucol inhibits carrageenan-induced edema and MPO and NAG activities in paw

Mice were treated with probucol (0.3, 1, 3 mg/kg, p.o.) or vehicle (Tween 80 5% in saline) 1 h before i.pl. administration of carrageenan (300 µg/paw). Paw edema was evaluated 1, 3 and 5 h after carrageenan injection. Carrageenan induced paw edema formation at all time points and probucol was able to reduce edema. The dose of 3 mg/kg of probucol inhibited by 44% carrageenan-induced edema, and this inhibition was maintained up to 5 h (39%). In contrast, lower doses of probucol did not affect carrageenan-induced paw edema (Fig. 3a). At 5 h, after the last paw edema measurement, samples of paw skin were collected for MPO and NAG activity assays. Probucol at 0.3 mg/kg (24%) and 3 mg/kg (36%) significantly inhibited carrageenan-induced MPO activity (Fig. 3b). In turn, probucol at 3 mg/kg (39%) but not at lower doses inhibited carrageenan-induced NAG activity (Fig. 3c). Probucol inhibited the carrageenan-induced MPO and NAG activities in a bell-shape curve (Fig. 3b-c). Given the results showed in figures 2 and 3, the dose of 3 mg/kg of probucol was chosen for the following experiments.

Probucol inhibits carrageenan-induced leukocyte recruitment into the peritoneal cavity

Mice were treated with probucol (3 mg/kg, p.o.) or vehicle (Tween 80 5% in saline) 1 h before i.p. administration of carrageenan (1 mg/cavity). Peritoneal exudates were harvested 5 h after carrageenan stimulus to evaluate leukocyte recruitment into the peritoneal cavity. Probucol at 3 mg/kg reduced total and polymorphonuclear leukocyte recruitment induced by carrageenan (41% and 43%, respectively) (Fig. 4a, b). Mononuclear cells were not recruited by carrageenan at this time point (Fig. 4c).

Probucol did not alter carrageenan-induced tissue antioxidant capacity

Mice were treated with probucol (3 mg/kg, p.o.) or vehicle (Tween 80 5% in saline) or naringenin (50 mg/kg, p.o.) 1 h before i.pl. administration of carrageenan (300 µg/paw). Paw skin samples were collected 3 h after carrageenan injection to evaluate ABTS free radical scavenging ability, FRAP ability and GSH levels. Carrageenan-induced oxidative stress was observed as a reduction of ABTS scavenging ability (44%), ferric reducing ability (FRAP assay, 35%) and GSH levels (35%). None of these parameters were reversed by the analgesic and anti-inflammatory dose of 3 mg/kg of probucol. However, naringenin, a well described antioxidant used as a positive control, restored tissue antioxidant capacity (Fig. 5).

Probucol reduces carrageenan-induced cytokine production and NF-κB activation

Mice were treated with probucol (3 mg/kg, p.o.) or vehicle (Tween 80 5% in saline) 1 h before i.pl. administration of carrageenan (300 µg/paw). Paw skin samples were collected 3 h after carrageenan injection and processed to evaluate TNF-α, CXCL1 and IL-β production, and phosphorylated and total NF-κB p65 subunit ratio. To evaluate peritoneal cytokine production and NF-κB activation, the same protocol was used except that carrageenan (1 mg/cavity) was injected i.p. and peritoneal exudates harvested after 5 h. Probucol inhibited carrageenan-induced TNF-α (62% and 44%), CXCL-1 (39% and 33%) and IL-1β (64% and 61%) production in the paw skin and peritoneal cavity, respectively (Fig. 6a, 6b, 6c, 6d, 6e and 6f respectively). In agreement with the cytokine production data, probucol also inhibited carrageenan-induced NF-κB activation in paw (39%) and in macrophages isolated from the peritoneal cavity (53%) as observed by a reduction of carrageenan-induced increase of p-NF-κB p65/NF-κB p65 ratio (Fig. 7a and 7b, respectively).

DISCUSSION

Inflammatory hyperalgesia is an outcome of a hierarchical release of cytokines and prostanoids. These mediators directly sensitize nociceptors in addition to further amplify the inflammatory response [45–47]. Therefore, these molecules including TNF-α, CXCL1, IL-1β, and signaling pathways involved in their production such as NF-κB, represent important therapeutic targets in the context of inflammatory diseases.

In the present work, we showed that probucol inhibited overt pain-like behavior induced by PBQ and acetic acid and mechanical and thermal hyperalgesia induced by carrageenan. These effects were accompanied by a reduction of neutrophil influx in the paw and in the peritoneal cavity. In these assessments (PBQ-induced abdominal writhings, carrageenan-induced mechanical hyperalgesia, MPO and NAG activities), probucol has shown a bell-shaped dose-response curve, i.e. the lowest and the highest doses of probucol were effective in oppose to the intermediate one.

Other chemicals were previously shown to display a similar dose-response pattern [48]. For instance, buprenorphine, an analgesic opioid, reduces pain through µ opioid receptors but, depending on the dose, it also activates Opioid Receptor-Like 1 (ORL-1) counteracting its antinociceptive effects [49]. In that sense, the bell-shaped dose response observed suggests probucol could target different molecules explaining why higher doses of probucol, e.g. 200 mg/kg, increase leukocyte migration whilst low doses inhibit cell recruitment [33]. In addition to reducing pain-like behavior and leukocyte recruitment, probucol at 3 mg/kg also showed antiedematogenic effects at all time points in the carrageenan model. Therefore, the 3 mg/kg dose exerts anti-nociceptive, anti-inflammatory and antiedematogenic effects altogether differently from clinically used therapies. For instance, at analgesic doses, the NSAIDs meloxicam and diclofenac did not inhibit paw edema in formalin-induced inflammation [50].

We evaluated the effect of probucol in carrageenan-induced oxidative stress since its beneficial effects are often attributed to its antioxidant properties [23–26]. However treatment with an analgesic/anti-inflammatory dose of probucol was not able to restore GSH levels or ROS scavenging ability in the carrageenan classic model of inflammation. A possible explanation for this discrepancy is that in the present study a single dose of probucol was used in contrast to longer periods of treatment in other studies observing antioxidant effects of probucol. For instance, Siveski-Iliskovic et al. (1994) and Colle et al. (2013) showed that probucol reduces MDA levels and augments endogenous antioxidants after a 2-week treatment with probucol using a cumulative dose of 60 mg/kg and

pretreatment with probucol in water for 2 months, respectively [24,26]. Al-Majed (2011) demonstrated increased GSH levels in a model of brain ischemia after treatment with 61 mg/kg/day of probucol for 7 days. In turn Park et al. (2007) showed that 2 doses of probucol at 30 mg/kg were unable to attenuate superoxide anion formation following acute ischemic brain injury in rats [35]. This evidence supports the present data that probucol exerts its analgesic and anti-inflammatory effects through mechanisms other than antioxidant properties in carrageenan model of acute inflammation. Importantly, the antioxidant, analgesic and anti-inflammatory flavonoid naringenin was used as control drug [19,39,51]. The inhibition of carrageenan-induced oxidative stress by naringenin treatment shows that the experimental protocol is well standardized and allows detecting antioxidant effects.

In our study, we showed that probucol inhibited carrageenan-induced TNF-α, CXCL1 and IL-1β production in the paw skin and peritoneal exudate. TNF-α, CXCL1 and IL-1β are cytokines involved in the hyperalgesia carrageenan-induced inflammation. Carrageenan induces TNF-α and CXCL1 that trigger IL-1β production, which in turn, induces prostaglandin E_2 (PGE_2) production [45]. PGE_2 induces nociceptor sensitization, which is detected as hyperalgesia [46,52]. Consistent with our present data, probucol was shown to inhibit IL-1β production *in vitro* [30,53–55] and TNF-α in models of atherosclerosis and type 2 diabetes [27,38]. In addition to sensitization of nociceptors, IL-1β and TNF-α are important mediators of leukocyte recruitment. They activate endothelial and immune cells and induce the release of chemoattractants for neutrophils, such as CXCL1 [56,57]. Neutrophil-dependent production of prostaglandins also contributes to nociception [46]. PGE_2 mediates thermal and mechanical hyperalgesia through PKC- and PKA-dependent mechanisms, respectively [58]. Probucol attenuates PGE_2 production by mesangial cells stimulated with oxLDL [59]. In addition, probucol reduces MAPK and PKC activities *in vitro* [60]. MAPK also play a role in TNF-α- and IL-1β-induced mechanical hyperalgesia [3,4], which depend on PGE_2 production [46]. Therefore targeting of PGE_2, PKC and MAPK could also underlie the mechanisms exerted by probucol.

The cascade of events culminating in carrageenan-induced hyperalgesia is NF-κB-dependent. Upon translocation to nucleus, NF-κB upregulates adhesion molecules on the surface of endothelial cells followed by transcription of COX-2 in recruited neutrophils [61,62]. We demonstrated that probucol inhibited NF-κB activation in the paw skin and in adherent macrophages from peritoneal exudates as observed by reduction of the p-NF-κB p65/total NF-κB p65 ratio. This finding is in agreement with previous studies showing that probucol reduces NF-κB translocation to the nucleus in endothelial cells treated with IL-1β [63], TNF-α [64], angiotensin II [65] or under hypoxic conditions [66]. Furthermore, long term treatment with probucol reduced protein levels of NF-κB in the left atrium tissue in rabbits [34]. Targeting NF-κB may explain the inhibitory effects of probucol in neutrophil influx. In fact, probucol was shown to reduce endothelial adhesiveness *in vitro* and to inhibit the expression of adhesion molecules [29,31,32,63,64]. For instance, in concentrations as low as 5 μM probucol inhibits expression of VCAM-1 but not ICAM-1 or E-selectin in endothelial cells treated with TNF-α [64]. Probucol at 50 μM inhibits expression of VCAM-1, ICAM-1 and E-selectin in endothelial cells treated with TNF-α or IL-1β [32,63]. In turn high doses of probucol (20 and 200 mg/kg) during 12 days increases leukocyte recruitment in diabetic rats, which present excessive oxidative stress driving leukocyte-endothelial interaction defects [33]. Thus, probucol inhibits pro-inflammatory cytokine production and adhesion molecules expression *in vitro* and in models of atherosclerosis

[37,38] and diabetes [27,34]. The present data show that probucol inhibits NF-κB activation and the consequent production of TNF-α, CXCL1 and IL-1β resulting in inhibition of carrageenan-induced hyperalgesia and leukocyte recruitment.

In conclusion, our findings show that probucol presents analgesic and anti-inflammatory activities by employing mechanisms other than its antioxidant properties. The present study demonstrates that these mechanisms involve inhibition of NF-κB activation and cytokine production showing, to our knowledge, for the first time that probucol inhibits inflammatory hyperalgesia.

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Figures

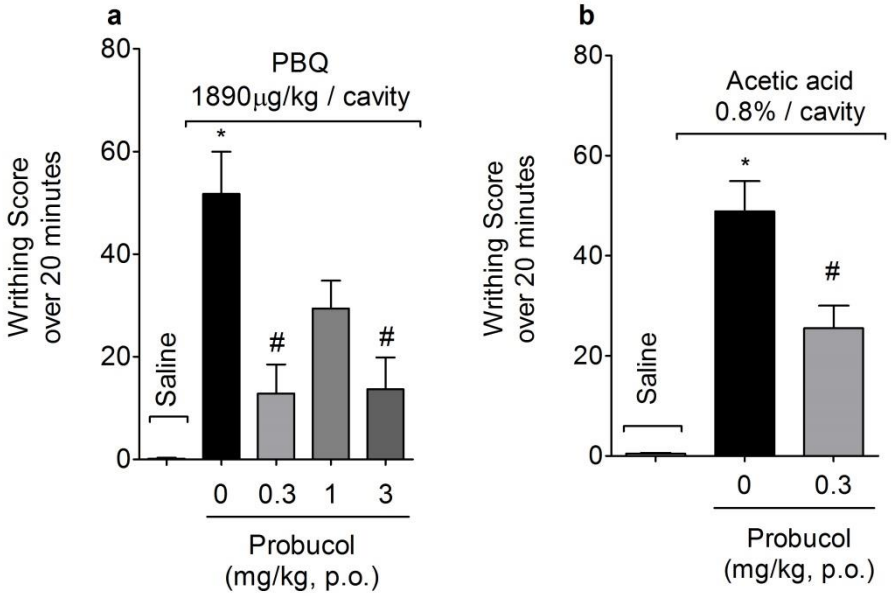

Fig. 1 Probucol inhibits overt pain-like behavior. Mice received probucol at doses 0.3, 1 and 3 mg/kg (p.o.) 1 h before i.p. injection of phenyl-p-benzoquinone (PBQ) (a) or acetic acid (b). The 0 mg/kg of probucol group stands for vehicle group. The number of writhings was evaluated 0–20 min after i.p. administration of PBQ or acetic acid. Results are expressed as mean ± SEM (n = 6 per group per experiment, representative of two separate experiments). One-way ANOVA followed by Tukey's post-hoc. *$p<0.05$ vs. saline group. #$p<0.05$ vs. vehicle group

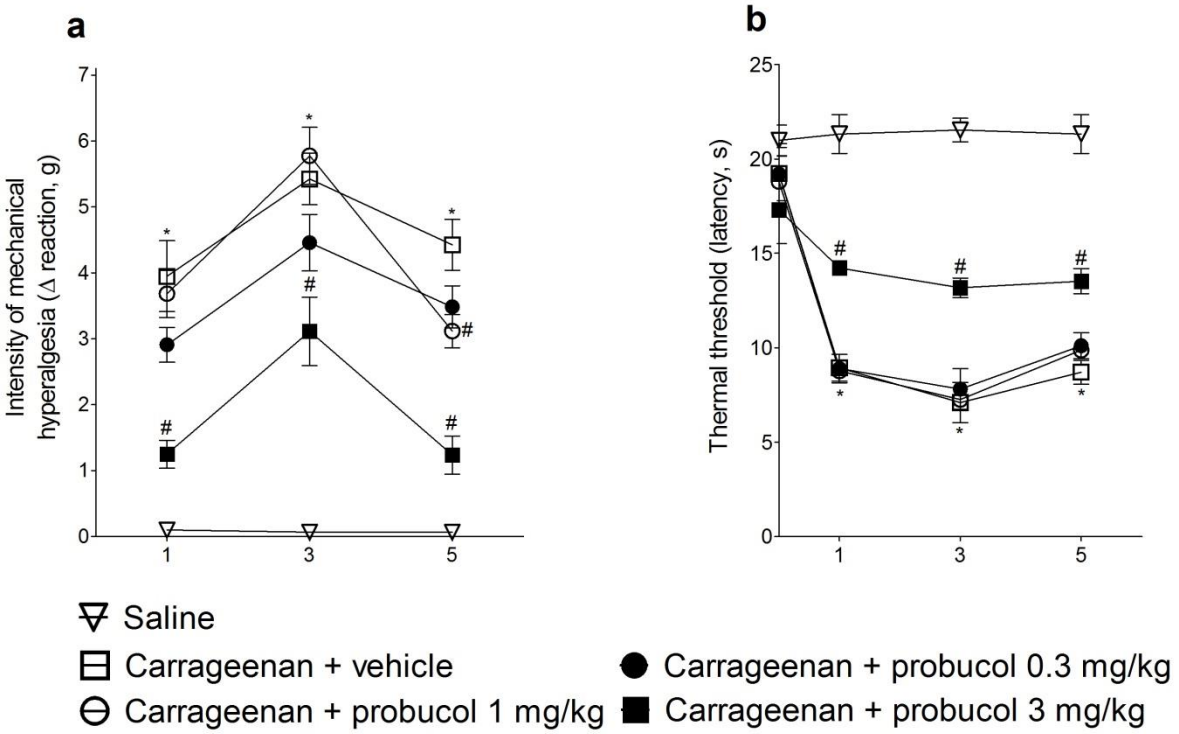

Fig. 2 Probucol inhibits carrageenan-induced mechanical and thermal hyperalgesia. Mice received probucol at doses 0.3, 1 and 3 mg/kg (p.o.) 1 h before i.pl. injection of carrageenan (300 µg/paw). Evaluation of mechanical (a) and thermal (b) were performed 1, 3 and 5 h after stimulus. Results are expressed as mean ± SEM (n = 6 per group per experiment, representative of two separate experiments). Two-way repeated measures ANOVA followed by Tukey's post-hoc. *p<0.05 vs. saline group. #p<0.05 vs. vehicle group.

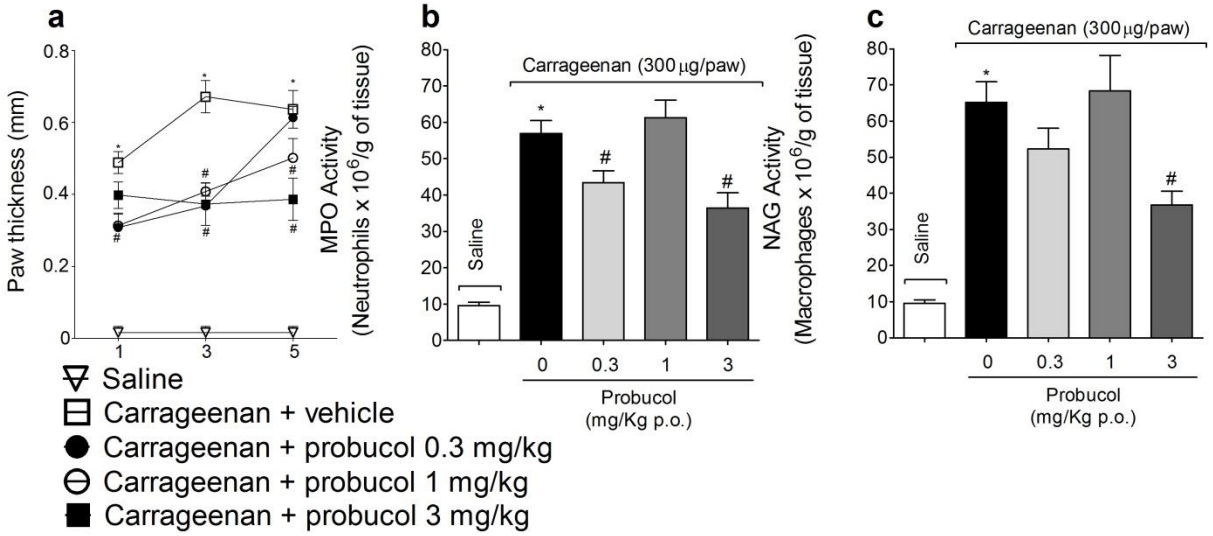

Fig. 3 Probucol inhibits carrageenan-induced paw edema, and MPO and NAG activities. Mice received probucol at doses 0.3, 1 and 3 mg/kg (p.o.) 1 h before i.pl. injection of carrageenan (300 µg/paw). Evaluation of edema was performed 1, 3 and 5 h after stimulus (a). MPO (b) and NAG (c) activities were assessed 5 h after carrageenan injection. Results are expressed as mean ± SEM (n = 6 per group per experiment, representative of two separate experiments). Two-way repeated measures ANOVA followed by Tukey's post-hoc was performed in Panel A. One-way ANOVA followed by Tukey's post-hoc was performed for Panels B and C. *p<0.05 vs. saline group. #p<0.05 vs. vehicle group.

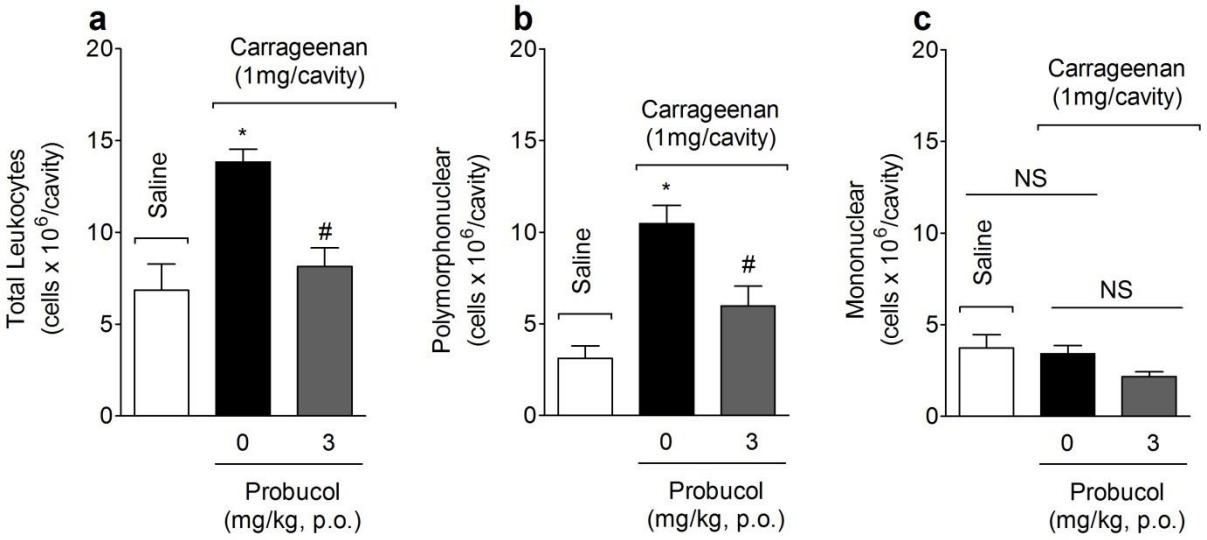

Fig. 4 Probucol inhibits carrageenan-induced leukocyte recruitment in the peritoneal cavity. Mice received probucol at 3 mg/kg (p.o.) 1 h before i.p. injection of carrageenan (1 mg/cavity). Total leukocytes (a) and differential counts of polymorphonuclear (b) and mononuclear (c) cells in peritoneal exudate were performed 5 h after stimulus. Results are expressed as mean ± SEM (n = 6 per group per experiment, representative of two separate experiments). One-way ANOVA followed by Tukey's post-hoc. *p<0.05 vs. saline group. #p<0.05 vs. vehicle group. NS stands for non-significant statistical differences (panel C).

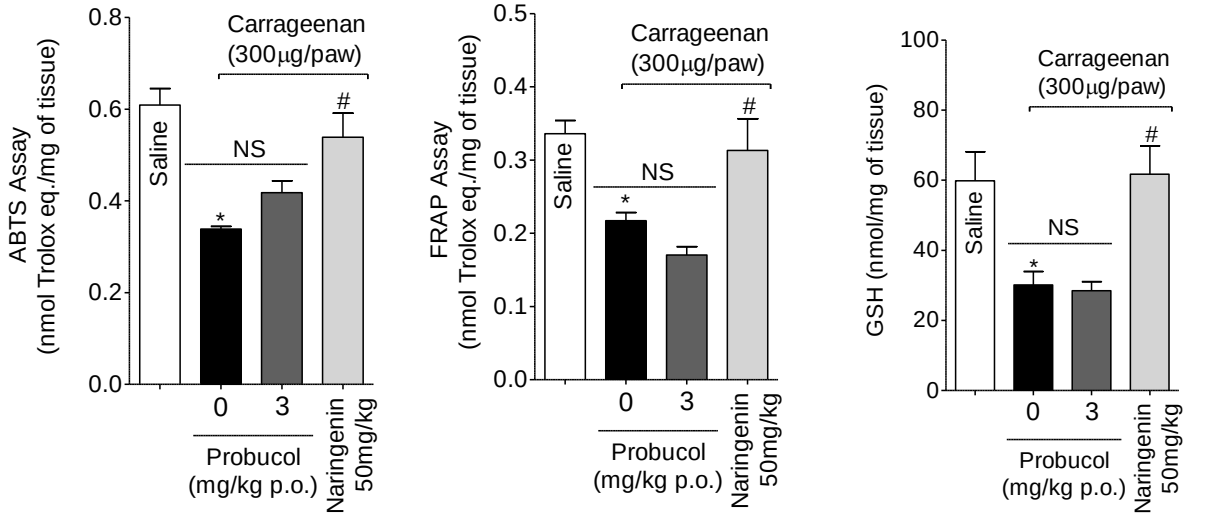

Fig. 5 Probucol does not affect carrageenan-induced oxidative stress. Mice received probucol at 3 mg/kg (p.o.) or the antioxidant naringenin at 50 mg/kg (p.o.) 1 h before i.pl. injection of carrageenan (300 μg/paw). ABTS and FRAP assays and GSH measurement were performed from paw skin samples removed 3 h post carrageenan injection. Results are expressed as mean ± SEM (n = 6 per group per experiment, representative of two separate experiments). One-way ANOVA followed by Tukey's post-hoc. *p<0.05 vs. saline group. #p<0.05 vs. vehicle group. NS stands for non-significant statistical differences.

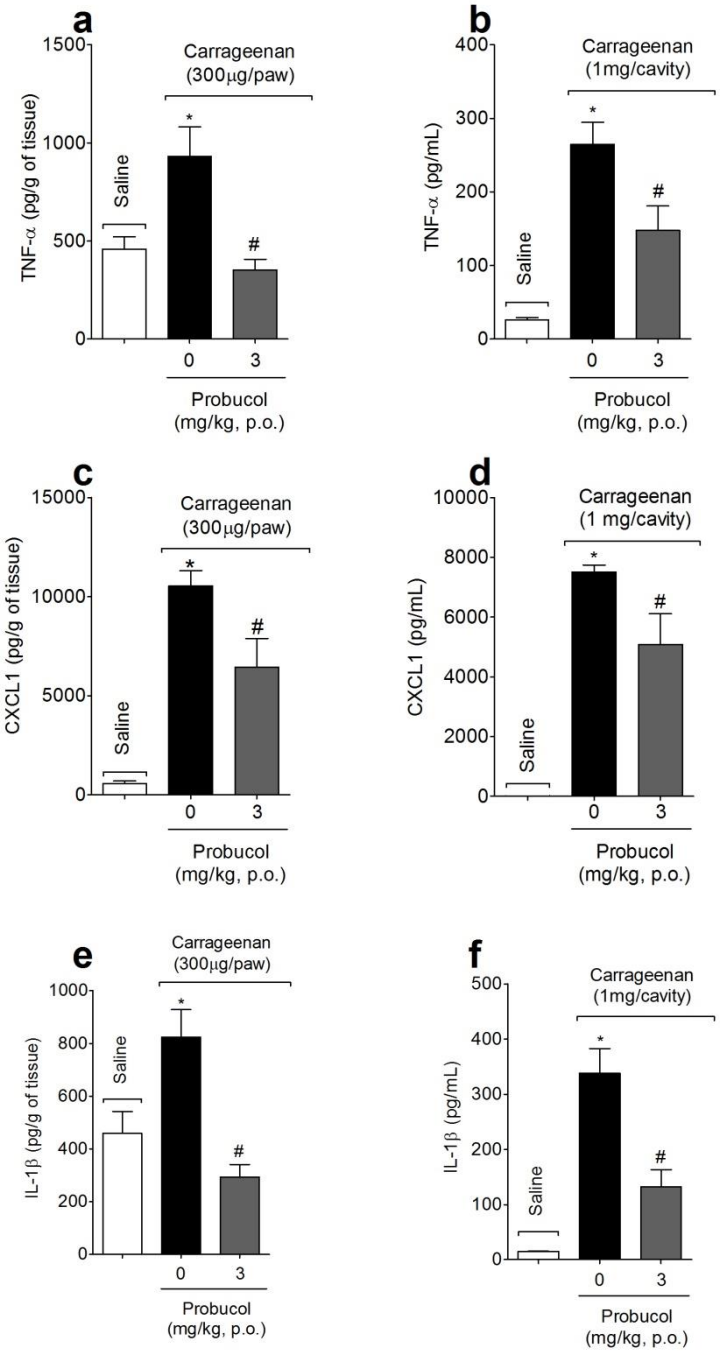

Fig. 6 Probucol inhibits carrageenan-induced cytokine secretion in the paw and peritoneal cavity. Mice received probucol at 3 mg/kg (p.o.) 1 h before i.pl. or i.p. injection of carrageenan (300 µg/paw or 1 mg/cavity) . Paw skin was removed 3 h post injection and peritoneal exudate was collected 5 h post-stimulus injection. TNF-α, CXLC1 and IL-1β levels were measured by ELISA. Results are expressed as mean ± SEM (n = 6 per group per experiment, representative of two separate experiments). One-way ANOVA followed by Tukey's post-hoc. *p<0.05 vs. saline group. #p<0.05 vs. vehicle group.

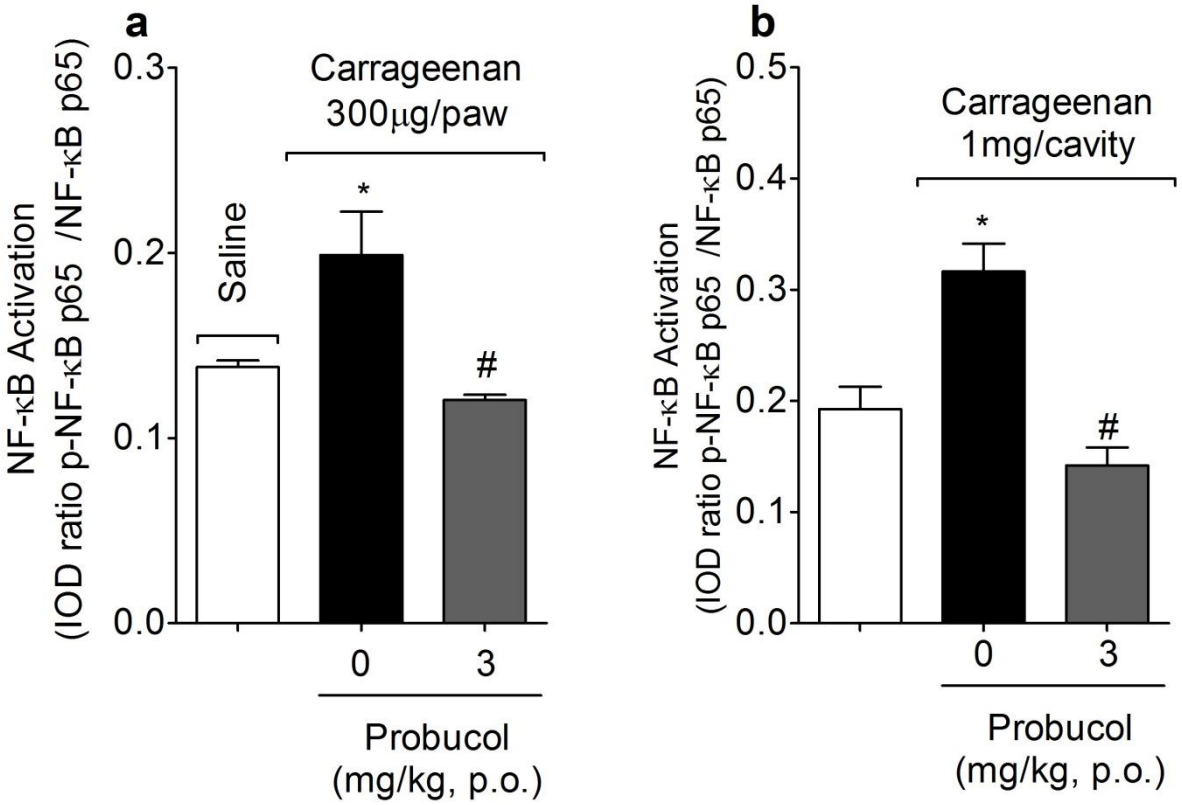

Fig. 7 Probucol inhibits carrageenan-induced NF-κB activation. Mice received probucol at 3 mg/kg (p.o.) 1 h before i.pl. injection of carrageenan in the paw (300 μg) or in the peritoneal cavity (1 mg). Paw skin was removed 3 h post-stimulus injection. Peritoneal exudates were harvested 3 h after carrageenan injection and adherent macrophages (4 h) were assayed. ELISA was used to determine the levels of total and phosphorylated NF-κB p65. Results are expressed as mean ± SEM (n = 6 per group per experiment, representative of two separate experiments). One-way ANOVA followed by Tukey's post-hoc. *$p<0.05$ vs. saline group. #$p<0.05$ vs. vehicle group.

4 ARTIGO PARA PUBLICAÇÃO (EUROPEAN JOURNAL OF PHARMACOLOGY)

O presente trabalho foi realizado na Universidade Estadual de Londrina, no Laboratório de Dor, Inflamação, Neuropatia e Câncer e foi submetido à revista European Journal of Pharmacology sob o título “Probucol attenuates lipopolysaccharide-induced inflammatory hyperalgesia by reducing NF-κB activity, cytokine production and leukocyte recruitment”.

Probucol attenuates lipopolysaccharide-induced leukocyte recruitment and inflammatory hyperalgesia by reducing NF-κB activation and cytokine production

Amanda Z. Zucoloto[a]; Marília F. Manchope[a]; Larissa Staurengo-Ferrari[a]; Felipe A. Pinho-Ribeiro[a,b]; Ana C. Zarpelon[a,c]; André L. L. Saraiva[d]; Tatiana N. Cecílio[d]; José C. Alves-Filho[d], Thiago M. Cunha[d], Gustavo B. Menezes[e], Fernando Q. Cunha[d], Rubia Casagrande[f], Waldiceu A. Verri, Jr.[a]

[a] Departamento de Ciências Patológicas, Centro de Ciências Biológicas, Universidade Estadual de Londrina, Rod. Celso Garcia Cid PR445 km 480, CEP 86057-970, Londrina, Paraná Caixa Postal 10.011, Brasil

[b] Department of Microbiology and Immunobiology, Division of Immunology, Harvard Medical School, Boston, MA 02115, USA

[c] Universidade Federal do Paraná, Campus Toledo, Rua General Rondon, 2195, 85.901-160, Toledo, Paraná, Brasil.

[d] Department of Pharmacology, Ribeirão Preto Medical School, University of São Paulo, Av. Bandeirantes 3900, CEP 14049-900, Ribeirão Preto, São Paulo, Brasil

[e] Departamento de Morfologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Avenida Presidente Antonio Carlos 6627, CEP 31270-901 Belo Horizonte, Minas Gerais, Brasil

[f] Departamento de Ciências Farmacêuticas, Centro de Ciências da Saúde, Universidade Estadual de Londrina, Av. Robert Koch, 60, CEP 86038-350, Londrina, Paraná, Brasil

*** Correspondence:**

Dr. Waldiceu A. Verri Jr.

waldiceujr@yahoo.com.br; waverri@uel.br; + 55 43 3371 4979

Abstract

Probucol is a synthetic molecule clinically used for prevention and treatment of hypercholesterolemia and atherosclerosis. Recent studies have shown that the beneficial effects of probucol mainly derive from its anti-inflammatory and antioxidant properties. Gram-negative bacteria are common infectious agents and their wall components, e.g. lipopolysaccharide (LPS), are important elicitors of inflammation. LPS is sensed by tissue resident cells and it triggers a TLR4/MyD88-dependent signaling cascade resulting in endothelial activation, leukocyte recruitment and nociception. Therefore the present study aimed to investigate the anti-inflammatory and analgesic effects of probucol in models of LPS-induced acute inflammation. Probucol at 0.3-30 mg/kg was administrated to male Swiss mice per oral 1 h before intraplantar or intraperitoneal lipopolysaccharide stimulus. Probucol at 3mg/kg reduced lipopolysaccharide-induced mechanical and thermal hyperalgesia. These effects were accompanied by reduced leukocyte influx and cytokine production in both paw skin and peritoneum exudate. Unexpectedly, probucol did not alter lipopolysaccharide-induced tissue oxidative stress at anti-inflammatory /analgesic dose. On the other hand, probucol inhibited lipopolysaccharide-induced NF-κB activation in paw tissue as well as NF-κB activity in cultured macrophages *in vitro,* reinforcing the inhibitory effect of probucol over the NF-κB signaling pathway. In this sense, we propose that probucol acts on resident immune cells, such as macrophages, targeting the NF-κB pathway. As a result, it prevents the amplification and persistence of the inflammatory response by attenuating NF-κB-dependent cytokine production and leukocyte recruitment explaining its analgesic effects as well.

Keywords: Acute Inflammation; Probucol; Pain; Nociception; LPS.

1 Introduction

Lipopolysaccharide (LPS) is one of the main constituents of Gram-negative bacteria cell wall and the prototypical agonist of Toll-like receptor 4 (TLR4), a pattern recognition receptor (PRR) expressed by many cell types including macrophages, monocytes and granulocytes (Nikaido, 2003; Vaure and Liu, 2014). Binding of LPS to TLR4 activates a downstream signaling cascade leading to activation and nuclear translocation of nuclear factor kappa B (NF-κB) followed by production of pro-inflammatory cytokines such as IL-1β, TNF-α, IL-6 and IFN-γ (Cho et al., 2016; Pinho-Ribeiro et al., 2016; Ruiz-Miyazawa et al., 2015b; Yuan et al., 2011). These mediators largely contribute to the amplification of the inflammatory response and its clinical manifestations. For instance, IL-1β and TNF-α activate endothelial cells favoring leukocyte recruitment in addition to modulation of immune cells and sensory neurons (Binshtok et al., 2008; Jin and Gereau IV, 2006; Oliveira et al., 2008; Wyble et al., 1997). Furthermore IFN-γ and IL-6 play important roles in macrophage modulation. Both cytokines induce differentiation of monocytes into macrophages (Chomarat et al., 2000; Munder et al., 1998). IL-6 is secreted by endothelial cells and promotes the switch from neutrophil to monocyte migration by increasing monocyte-attracting chemokines and reducing neutrophil-attracting ones (Hurst et al., 2001; Kaplanski et al., 2003). In addition to its pro-inflammatory properties an increasing number of studies have shown the participation of IL-6 in pain signaling (Cunha et al., 1992;Murphy et al., 1999; Yang et al., 2015).

Infectious diseases are a major public health concern and gram-negative bacteria arise as important causative agents of nosocomial infections (Ho et al., 2010). The host immune response and the use of antibiotics culminate in the release of large amounts of LPS (Ginsburg and Koren, 2008). In turn LPS contributes to disease morbidity by triggering pain signaling in a TLR4-dependent manner (Calil et al., 2014). In that sense, targeting TRL4 downstream signaling such as the NF-κB pathway is of great importance in preventing deleterious effects of Gram-negative bacterial infections. Therefore molecules presenting anti-inflammatory and analgesic properties emerge as candidates for treating LPS-induced inflammation and hyperalgesia.

Probucol is a synthetic compound clinically used for the treatment of hypercholesterolemia and atherosclerosis. Its anti-inflammatory and antioxidant effects have been shown in experimental models of diabetes, neurodegenerative diseases and brain ischemia (Al-Majed, 2011; Fu et al., 2015; Li et al., 2011; Park et al., 2007; Siveski-Iliskovic et al., 1994). For instance probucol

inhibits production of pro-inflammatory cytokines IL-1β, TNF-α and IL-6 *in vivo* and *in vitro* (Jung et al., 2016; Ku et al., 1990, 1988; Li et al., 2011; Liu et al., 2000; Zhang et al., 2009). Additionally probucol was shown to attenuate NF-κB activation in endothelial cells (Aoki et al., 2001; Chang et al., 2010; Chen et al., 2003; Zhang et al., 2013). Overall these findings support the beneficial effects of probucol in experimental models of inflammation-driven diseases. Given that the inflammatory response evoked by gram-negative bacteria strongly contribute to disease morbidity, this study aimed to evaluate the effects of probucol in LPS-induced acute inflammatory pain and leukocyte recruitment.

2 Materials and methods

2.1 Animals

Male Swiss (20-25g) from Universidade Estadual de Londrina, Parana, Brazil and Lysm-eGFP mice (20-25g) kindly provided by Ribeirão Preto Medical School were used in this study. Mice were housed in standard clear plastic cages, and received food and water *ad libitum* under a 12:12 h light/dark cycle at 21 °C. All behavior testing were performed between 9 am and 5 pm in temperature-controlled room. The Animal Welfare and Ethics Committee of Londrina State University approved this study (process number 1012.2015.74). All efforts were made to minimize the number of animals used and their suffering.

2.2 Drugs

Materials were obtained from the following sources: LPS from *Escherichia coli* 0111:B4 (Santa Cruz Biotechnology, Dallas, TX, USA), Naringenin (Santa Cruz Biotechnology, Dallas, TX, USA), Probucol (Santa Cruz Biotechnology, Dallas, TX, USA), Rhodamine 6G (Santa Cruz Biotechnology, Dallas, TX, USA).

2.3 Experimental procedures

Mice were treated with probucol (0.3, 3, 30 mg/kg, per oral) or vehicle (Tween 80 5% in saline) 1 h before the administration of LPS. Leukocyte recruitment into the peritoneal cavity was assessed 6 h after i.p. administration of LPS (200 ng/200 µL/cavity). Mechanical hyperalgesia and thermal hyperalgesia were evaluated 1, 3 and 5 h after i.pl. administration of LPS (200 ng/20 µL). Paw skin was collected for assessment of leukocyte recruitment by myeloperoxidase (MPO) and *N*-acetylglucosaminidase (NAG) activity assays 5 h after LPS injection. ABTS (2,2'-Azinobis-3-ethylbenzothiazoline 6-sulfonic acid), GSH (reduced glutathione), NBT (Nitroblue tetrazolium)

and TBARS (Thiobarbituric Acid Reactive Substances) assays were performed using paw skin collected 3 h after LPS injection. IL-1β, TNF-α, IL-6 and IFN-γ levels were also evaluated 3 h after LPS injection in the paw or 6 h post-LPS injection in the peritoneum exudate by ELISA. Phosphorylated and total NF-κB ratio (activation) was evaluated 3 h after LPS injection in the paw by ELISA kits. Doses of stimulus and time points of sample collection were based on previous studies in our laboratory (Pinho-Ribeiro et al., 2016; Ruiz-Miyazawa et al., 2015a). Confocal microscopy was performed to evaluate neutrophil recruitment 4 h after LPS intraperitoneal stimulus (Marques et al., 2015). RAW 264.7 macrophages kindly provided by Ribeirão Preto Medical School were treated with probucol (0.05-5 μM) 1 h prior to exposure to LPS (1 μg/mL). Six hours after LPS stimulus, supernatants and cell lysates were obtained to evaluate IL-1β, TNF-α, IL-6 and IFN-γ levels by ELISA, and NF-κB activity was also evaluated in RAW 264.7 macrophages by luciferase gene reporter assay.

2.4 Mechanical hyperalgesia test

Mechanical hyperalgesia was assessed by an electronic version of von Frey filaments (Cunha et al., 2004). A handheld force transducer (electronic anesthesiometer; Insight, Ribeirao Preto, SP, Brazil) adapted with a 0.5 mm^2 polypropylene tip was used to evoke hind paw nociceptive withdrawal response. The intensity of the pressure (in g) at the moment of paw withdrawal was automatically recorded. The mechanical threshold was tested before (baseline) and after LPS i.pl. administration (200 ng/paw). The results are expressed as delta (Δ) withdrawal threshold (in g), obtained by subtracting the measurements at each time point (1, 3 and 5 h after i.pl. LPS injection) from the baseline values.

2.5 Thermal hyperalgesia test

Animals were place on a hot plate apparatus (Insight, Ribeirao Preto, SP, Brazil) at a constant temperature of 52 °C. The end-point was characterized by removal of the paw followed by flinching or licking the paw, and latency time until the end-point reaction was determined (Kuraishi et al., 1983). Maximum latency until end-point was set at 25 s to avoid tissue damage. The results are expressed as means of latency (s) within experimental groups.

2.6 MPO and NAG activity assays

MPO and NAG kinetic-colorimetric assays were performed as indirect indicators of neutrophil and macrophage recruitment to paw tissue, respectively. Paw skin samples were collected 5 h after LPS injection and homogenized in ice-cold K_2HPO_4 buffer (400 μL, 50 mM, pH 6.0)

containing HTAB (0.5% weight/volume) using a tissue-tearor (Biospec, Bartlesville, OK, USA). Samples were centrifuged (16100 *g* x 2 min x 4 °C) and the supernatants were used for both assays. The MPO activity assay was performed as previously described (Bradley and Christensen, 2016). Briefly, 50 mM phosphate buffer, pH 6.0 containing 0.167 mg/ mL o-dianisidine dihydrochloride and 0.015 % hydrogen were added to samples and the absorbance was read at 450 nm (Multiskan GO Microplate Spectrophotometer, Thermo Scientific, Vantaa, Finland). The values obtained were compared to a standard curve of neutrophils and results are expressed as MPO activity (neutrophils x 10^6 / g of tissue). The NAG activity assay was performed as previously described (Barcelos et al., 2005). Samples were diluted in citrate/phosphate buffer (0.1 M citric acid, 0.1 M Na_2HPO_4, pH 4.5) in a proportion of 5:1. p-nitrophenyl-N-acetyl-β-glucosaminide dissolved in citrate/phosphate buffer was added to samples followed by addition of 0.2 M glycine buffer (pH 10.6). The absorbance was read at 405 nm (Multiskan GO, Thermo Scientific). The values obtained were compared to a standard curve of macrophages and results are expressed as NAG activity (macrophages x 10^6 / g of tissue).

2.7 Leukocyte recruitment into the peritoneal cavity

Leukocyte recruitment into the peritoneal cavity was assessed 6 h after i.p. administration of LPS (200 ng/cavity). The dose of the inflammatory stimulus was based on previous studies performed in our laboratory (Pinho-Ribeiro et al., 2016; Ruiz-Miyazawa et al., 2015a). Peritoneal cavities were washed with 1 mL of phosphate-buffered saline (PBS). Total leukocyte counts were performed after dilution of peritoneal exudate in Turk solution (2% acetic acid) using a Neubauer chamber. Differential cell counts were performed using the Fast Panoptic Kit for histological analysis (Laborclin, Pinhas, BR, Brazil) to distinguish polymorphonuclear (PMNs) from mononuclear cells under a light microscope (Olympicus Optical Co., Hamburg, Germany). The results are expressed as number of cells x 10^6 per cavity.

2.8 Total Antioxidant Capacity

Antioxidant capacity was assessed by the ABTS assay. This test was adapted to a 96-well microplate format as previously described (Navarro et al., 2013). Paw skin samples were collected 3 h after i.pl. stimulus (LPS 200 ng/paw) and homogenized with a tissue-tearor in ice-cold KCl buffer (500 µL, 1.15% w/v). Samples were centrifuged at 835 *g* at 4 °C for 10 min, and supernatants were used. ABTS assay was performed as an indicator of the sample's ability to scavenge the free radical ABTS. Diluted ABTS solution was added to samples and the

absorbance was measured at 730 nm after 6 min of incubation at 25 °C (Multiskan GO, Thermo Scientific). The results were equated against a standard Trolox curve (0.02-20 nmol) and expressed as nmol Trolox eq. per mg of tissue.

2.9 GSH levels measurement

Paw skin samples were collected 3 h after i.pl. stimulus (LPS 200 ng/paw) and stored at -80 °C for at least 48 hours. For the assay, samples were homogenized with a tissue-tearor in 200 µL of 0.02 M EDTA after thawing. Trichloroacetic acid 50% solution was added to homogenates and the mixture was homogenized for 15 min using a vortex followed by centrifugation (1500 *g* x 15 min x 4 °C). The supernatants were added to a 96-well microplate followed by addition of 0.2 M TRIS buffer, pH 8.2, and 0.01 M DTNB. The absorbance was measured at 412 nm (Multiskan GO, Thermo Scientific) after 5 min incubation at room temperature. The values obtained were compared to a standard curve of GSH. The results are expressed as GSH levels (nmol) per mg of tissue (Navarro et al., 2013).

2.10 Superoxide anion production

Superoxide anion production was assessed by the NBT assay. Paw skin samples were collected 3 h after i.pl. stimulus (LPS 200 ng/paw). Samples were homogenized with a tissue-tearor in 500 µL of ice-cold KCl buffer (1.15% w/v) and the homogenates were used for the assay. The test was adapted to a microplate as previously described (Hohmann et al., 2013). The NBT reduction was measured at 600 nm (Multiskan GO, Thermo Scientific). The results are expressed as optical density (OD) per mg of tissue.

2.11 Lipid peroxidation

Lipid peroxidation was measured by the TBARS (Thiobarbituric Acid Reactive Substances) assay. Paw skin samples were collected 3 h after i.pl. stimulus (LPS 200 ng/paw) and homogenized with a tissue-tearor in ice-cold KCl buffer (500 µL, 1.15% w/v). The test was adapted to a microplate as previously described (Hohmann et al., 2013). The intermediate product of lipid peroxidation malondialdehyde (MDA) was determined by subtracting the absorbance at 535 nm from the absorbance at 572 nm Multiskan GO, Thermo Scientific). The results are expressed as nmol of MDA per mg of tissue.

2.12 Cytokine measurement

Paw skin samples were collected 3 h after stimulus (LPS 200 ng/paw). Samples were homogenized with a tissue-tearor in 500 µL of ice-cold buffer with protease inhibitors followed

by centrifugation (835 *g* x 15 min x 4 °C). Peritoneal exudates were collected using a micropipette after washing the peritoneal cavity with 1mL of phosphate-buffered saline (PBS) 6 h after stimulus (LPS 200 ng/cavity.) The supernatants of paw skin homogenates and peritoneal exudates were used to determine IL-1β, TNF-α, IL-6 and IFN-γ levels by enzyme-linked immunosorbent assay (ELISA) using commercial kits (eBioscience, San Diego, CA, USA). Absorbance was measured at 450 nm (Multiskan GO, Thermo Scientific). The results are expressed as picograms of cytokines per 100 mg of tissue or mL.

2.13 NF-κB activation

Paw skin samples were collected 3 h after stimulus (LPS 200 ng/paw). Samples were homogenized with a tissue-tearor in 400 µL of ice-cold lysis buffer (Cell Signaling, Danvers, MA, USA) followed by centrifugation (16100 *g* x 10 min x 4 °C). The supernatants were used for determination of phosphorylated NF-κB p65 subunit and total NF-κB p65 subunit levels using ELISA PathScan Kits (Cell Signaling, Danvers, MA, USA) according to the manufacturer's directions. Absorbance was measured at 450 nm (Multiskan GO, Thermo Scientific). The results are expressed as IOD ratio (p-NF-κB p65/total NF-κB p65).

2.14 Immunofluorescence

Lysm e-GFP mice were euthanized with isoflurane 4 h after LPS stimulus (200 ng/cavity). A midline laparotomy was performed to remove the mesentery, which was placed on a stage as previously described (Marques et al., 2015). Rhodamine 6G was administrated intravenously 5 minutes before euthanasia for background staining. Imaging was performed using a confocal microscope (Leica TCS SP8, Leica, Wetzlar, Germany) with a 40x long distance objective. Images were processed using Leica EL6000 software (Leica, Wetzlar, Germany). The results are expressed as number of neutrophils per mm^3 of tissue.

2.15 Cell culture

RAW 264.7 murine macrophages were routinely cultured in RPMI medium supplemented with 10% fetal bovine serum and penicillin-streptomycin at 37°C, 5% CO_2. To evaluate the effect of probucol *in vitro*, RAW 264.7 macrophages culture in 24-well plates for 24 h prior to experiments ($3x10^5$ cells per well). Cells were then treated with probucol (0.05-5 µM) for 1 h before stimulation with LPS (1 µg/mL). Pro-inflammatory cytokines IL-1β, TNF-α and IL-6 were measured by ELISA in the supernatants collected 6 h after stimulus. The results are expressed as pg per mL. After determining the ideal concentration of probucol (5 µM), RAW 264.7 murine

macrophages stably expressing luciferase on the NF-κB responsive promoter (pNF-κB-Luc) were cultured as described above. The intracellular contents were obtained by washing the adherent cells with lysis buffer (TNT). The luciferase activity in cell lysates was determined on a luminometer (Victor X5, PerkinElmer, Waltham, MA) using the Dual Luciferase Reporter assay system (Promega, WA, USA). The results are expressed as a ratio of relative luminescence units (RLU). Lactate dehydrogenase (LDH) leakage was measured in supernatants to evaluate cytotoxicity using the LDH Cytotoxicity Assay Kit (Cayman Chemical Company, MI, USA). The assay and data analysis were performed according to the manufacturer's directions. The results are expressed as % of LDH leakage. LDH levels in lysates from the same cell concentration, i.e. $3x10^5$ cells /well, were considered 100% of LDH release.

2.16 Statistical analyses

Results are presented as mean ± SEM of measurements made on 6 mice per group per experiment, and are representative of two independent experiments. Two-way repeated measures analysis of variance (ANOVA) followed by Tukey's post hoc were used to compare groups and doses at all time points when responses were measured at different time points after stimulus injection (mechanical and thermal hyperalgesia tests). One-way ANOVA followed by Tukey's post-hoc was performed for data from single time point experiments. $P < 0.05$ was considered statistically significant. All data analyses were performed using GraphPad Prism® 5.0 (GraphPad Software, Inc., USA-500.288), as well as elaboration of figures. Schematic representations were elaborated using Servier and Somersault 18:24 free databases of scientific illustrations.

3 Results

3.1 Probucol inhibits LPS-induced leukocyte recruitment into the peritoneal cavity

Mice were treated with probucol (0.3, 3 or 30 mg/kg, p.o.) or vehicle (Tween 80 5% in saline) 1 h before i.p. administration of LPS (200 ng/cavity). Peritoneal exudates were harvested 6 h after LPS stimulus to evaluate leukocyte recruitment into the peritoneal cavity. Probucol at 0.3 mg/kg did not inhibit total, polymorphonuclear or mononuclear leukocyte recruitment (Fig. 1). In turn probucol at 3 and 30 mg/kg reduced total leukocyte recruitment induced by LPS (61% and 69%, respectively) as well as polymorphonuclear (62% and 69%, respectively) and mononuclear (57% and 73%, respectively) cell migration to the peritoneal cavity (Fig. 1). Since no statistical differences were observed between these two doses, probucol at 3 mg/kg was chosen for the

following *in vivo* experiments. To further confirm the effect of probucol on neutrophil recruitment, Lysm-eGFP mice were treated with probucol at 3 mg/kg, p.o. or vehicle (Tween 80 5% in saline) 1 h before i.p. administration of LPS (200 ng/cavity). The mesentery was removed 4 h later and imaging analysis was performed to evaluate neutrophil recruitment. Probucol at 3 mg/kg inhibited 75% of LPS-induced neutrophil recruitment (Fig. 2)

3.2 Probucol inhibits LPS-induced mechanical and thermal hyperalgesia as well as neutrophil and macrophage recruitment in the paw skin

Mice were treated with probucol at 3 mg/kg, p.o. or vehicle (Tween 80 5% in saline) 1 h before i.pl. administration of LPS (200 ng/paw). Mechanical and thermal hyperalgesia were evaluated at 1, 3 and 5 h after LPS injection. LPS induced mechanical and thermal hyperalgesia at all times points ($p < 0.05$). Probucol at 3 mg/kg reduced mechanical hyperalgesia 3 and 5 h after LPS (up to 44%) (Fig. 3a). In turn, probucol inhibited LPS-induced thermal hyperalgesia at all time points, up to 91%. Thermal threshold was not statistically different from saline group at 3 h and 5 h ($p > 0.05$) (Fig. 3b). In addition, probucol at 3 mg/kg has reduced cell recruitment in the paw. Paw tissue was removed 5 h after LPS stimulus (200 ng/paw) and the samples were used to evaluate MPO and NAG activities as markers of neutrophil and macrophage recruitment, respectively. Probucol reduced 59% of LPS-induced MPO activity (Fig. 3c) and 65% of NAG activity (Fig. 3d).

3.3 Probucol does not inhibit LPS-induced ROS production in the paw skin

Mice were treated with probucol at 3 mg/kg, p.o. or vehicle (Tween 80 5% in saline) 1 h before i.pl. administration of LPS (200 ng/paw). Another group received the antioxidant naringenin at 50 mg/kg 1 h before LPS stimulus (Pinho-Ribeiro et al., 2016). Paw skin samples were collected 3 h after LPS injection to evaluate the following parameters of oxidative stress: ABTS free radical scavenging ability, GSH levels, lipid peroxidation by TBARS and superoxide anion formation by NBT reduction. LPS induced tissue oxidative stress observed as reduction of ABTS scavenging ability (35%) and GSH levels (64%) as well as increase of lipid peroxidation (TBARS assay, 58%) and superoxide anion formation (NBT reduction, 330%) compared to the saline group. Naringenin at 50 mg/kg reversed these effects ($p < 0.05$) (Fig. 4). In turn, probucol at 3 mg/kg neither restored tissue antioxidant capacity nor reduced ROS formation ($p > 0.05$) (Fig. 4a-d).

3.4 Probucol inhibits LPS-induced cytokine production in the paw skin and peritoneal cavity

Mice were treated with probucol at 3 mg/kg, p.o. or vehicle (Tween 80 5% in saline) 1 h before i.pl. or i.p. administration of LPS (200 ng/paw). Paw skin samples were removed 3 h post-LPS injection and peritoneal exudates were harvested after 6 h. Samples were processed and homogenized to evaluate TNF-α, IL-6, IL-1β and IFN-γ production by ELISA. Probucol inhibited LPS-induced TNF-α (53% and 54%), IL-6 (40% and 62%), IL-1β (31% and 47%) and IFN-γ (33% and 97%) production in the paw skin and peritoneal cavity, respectively (Fig. 5a-h).

3.5 Probucol inhibits LPS-induced cytokine production in macrophages

RAW 264.7 murine macrophages were treated with probucol (0.05-5 μM) 1 h before stimulation with LPS (1 μg/mL). The supernatants were collected after 6 h and the pro-inflammatory cytokines IL-1β, TNF-α and IL-6 were measured. Probucol at 0.05 μM only inhibited LPS-induced IL-6 levels (36%). The intermediate concentration, 0.5 μM, inhibited the secretion of both IL-6 (52%) and IL-1β (63%). In turn probucol at 5 μM inhibited LPS-induced TNF-α (39%) along with IL-6 (92%) and IL-1β (67%) production (Fig. 6a-c). Thus, this concentration was chosen to further evaluate whether probucol modulates NF-κB activity *in vitro*.

3.6 Probucol inhibits LPS-induced NF-κB activation in the paw skin and NF-κB activity in RAW 264.7 macrophages

Mice were treated with probucol at 3 mg/kg, p.o. or vehicle (Tween 80 5% in saline) 1 h before i.pl. administration of LPS (200 ng/paw). Paw skin samples were collected 3 h post-LPS injection. Samples were processed and homogenized to evaluate NF-κB activation by ELISA. Probucol inhibited LPS-induced NF-κB activation in paw tissue (39%) demonstrated by a reduction of LPS-induced increase of p-NF-κB p65/NF-κB p65 ratio (Fig. 7a). RAW 264.7 murine macrophages expressing luciferase on the NF-κB responsive promoter (pNF-κB-Luc) ($3x10^5$ cells per well) were treated with probucol at 5 μM 1 h before stimulation with LPS (1 μg/mL). Cell lysates were obtained 6 h after LPS stimulus. NF-κB activity was evaluated by measurement of luciferase activity using the Dual Luciferase Reporter assay system (Promega, WA, USA). Probucol inhibited LPS-induced NF-κB activity (81%) in RAW 264.7 macrophages (Fig. 7b). Treatment with probucol at 0.05-5 μM did not increase LDH leakage indicating its effects (inhibition of LPS-induced cytokine production and NF-κB activity) were not due to increased cell death (data not shown). This corroborates with previous studies showing that

probucol at 1 μM and up to 150 μM does not affect cell viability in BV2 microglial cells (Jung et al., 2016) or endothelial cells (Chang et al., 2010; Chen et al., 2003).

4 Discussion

Pathogens are first sensed by innate immune cells through PRR-dependent recognition of conserved surface molecules generically named pathogen-associated molecular patterns (Medzhitov, 2001). Tissue resident macrophages play a crucial role in pathogen recognition and subsequently endothelial activation and cell recruitment (Medzhitov, 2008). Neutrophils, the first cells recruited to the infected tissue, display a wide range of microbicidal mechanisms, such as phagocytosis, degranulation of antimicrobial molecules and formation of NETs (Kolaczkowska and Kubes, 2013). These events briefly summarize the initial immune response evoked to clear the infectious agent. Cellular necrosis and sterile inflammatory signals also activate PRR accounting to the inflammatory response, which can cause tissue lesion (Chen and Nuñez, 2010). Therefore, the fine-tuning between the necessary protective inflammatory response and excessive inflammation causing tissue lesion is essential. Later, mechanisms of resolution counteract the acute inflammatory response in order to repair tissue damage and reestablish tissue homeostasis (Serhan et al., 2008). Nonetheless, the persistence of PAMPs in the inflammatory milieu further activates macrophages and neutrophils sustaining the inflammatory response. For instance neutrophil-derived mediators and antibiotics promote bacteriolysis leading to release of bacterial components such as LPS. In turn, LPS and other bacterial PAMPs boost the immune response contributing to the pathophysiology of severe health conditions such as septic shock (Ginsburg, 2002; Ginsburg and Koren, 2008).

In the present work, we show that probucol inhibited neutrophil and monocyte recruitment in the peritoneal cavity and paw tissue as well as NF-κB activation and cytokine production. Despite previous studies reporting the antioxidant properties of probucol in different disease models, we have not observed such effect at the analgesic / anti-inflammatory doses tested (Al-Majed, 2011; Colle et al., 2013; Siveski-Iliskovic et al., 1994). We attribute this discrepancy to the use of lower doses of probucol administrated in a single treatment opposing to long-term treatments suggesting that probucol displays antioxidant effects in cumulative doses *in vivo*. In agreement with the present data, in a model of acute brain ischemia, two treatment with probucol at 30 mg/kg, a dose also used in our work, did not reduce superoxide anion production (Park et al.,

2007). Therefore, our results suggest that probucol presents anti-inflammatory effects independently of its antioxidant properties in LPS-induced inflammation.

Probucol at 3 mg/kg inhibited LPS-induced leukocyte recruitment and hyperalgesia. The mechanisms underlying these effects could be attributed to inhibition of NF-κB activation and cytokine production. LPS triggers NF-κB activation in a TLR4/MyD88-dependent manner leading to the release of TNF-α followed by CXCL1 and IL-1β resulting in mechanical hyperalgesia. Further, the LPS-induced hyperalgesia is independent on TLR4/TRIF signaling (Calil et al., 2014). Probucol was shown to inhibit IL-1β- and TNF-α-induced NF-κB translocation to the nucleus in endothelial cells (Chang et al., 2010; Chen et al., 2003). More recently, Jung et al. (2016) demonstrated the inhibitory effect of probucol over NF-κB by assessing its activity and nuclear translocation. In their study, HEK 293-T cells were treated with probucol at 5 μM 3 h prior exposure to LPS (Jung et al., 2016). The present work showed that probucol inhibited LPS-induced NF-κB activation in the paw skin as observed by reduction of the p-NF-κB p65/total NF-κB p65 ratio. In addition, we have observed a significant reduction in NF-κB activity in probucol-treated RAW 264.7 macrophages stimulated with LPS. In agreement, probucol reduced TNF-α and IL-β levels in both paw tissue and peritoneal cavity as well as in macrophage culture.

TNF-α and IL-β directly mediate leukocyte recruitment. These mediators act on endothelial cells through their respective receptors (TNF Receptor 1 and IL-1 Receptor) activating the NF-κB canonical pathway and upregulating adhesion molecules such as E-selectin and ICAM-1 (Wyble et al., 1997, 1996). The activated endothelium favors neutrophil influx, which was previously shown to be dependent on TLR4/MyD88 signaling in the paw tissue (Calil et al., 2014) and knee joint (Guerrero et al., 2016) in the presence of LPS. In our study, we demonstrated that probucol at 3 mg/kg reduced neutrophil recruitment in the paw tissue by measuring myeloperoxidase activity. In the peritoneal cavity, we also observed a reduction in neutrophil influx by differential cell count and we further confirmed this result by performing immunofluorescence in the mesentery of Lysm-eGFP mice after LPS i.p. injection. Mononuclear recruitment was also inhibited by probucol in both paw tissue and the peritoneal cavity, as demonstrated by NAG activity assay and differential cell count, respectively. This could be partially attributed to probucol-mediated inhibition of IL-6 and IFN-γ production in the paw tissue and peritoneal cavity. IL-6 plays a role in monocyte migration by increasing endothelial-derived monocyte-

attracting chemokines and, along with IFN-γ, IL-6 promotes macrophage differentiation (Chomarat et al., 2000; Hurst et al., 2001; Kaplanski et al., 2003; Munder et al., 1998). Additionally, probucol was previously shown to inhibit the expression of adhesion molecules by endothelial cells. Low concentrations of probucol (5 μM) inhibited VCAM-1 expression whilst intermediate or higher concentrations (up to 50 μM) were required to inhibit ICAM-1 and E-selectin in endothelial cells stimulated with TNF-α or IL-1β (Chang et al., 2010; Chen et al., 2003; Zapolska-Downar et al., 2000). Our results, in agreement with previous studies, support the hypothesis that probucol exerts its anti-inflammatory effects by targeting the NF-κB pathway. By inhibiting NF-κB activation probucol also impairs the production of its downstream targets, e.g. pro-inflammatory cytokines, culminating in reduced leukocyte recruitment.

We have also demonstrated the analgesic potential of probucol in LPS-induced thermal and mechanical hyperalgesia. Pain is a clinical symptom commonly reported by patients with infectious diseases (Ho et al., 2010; Wadachi and Hargreaves, 2006). LPS evokes inflammatory hyperalgesia by triggering a cytokine cascade dependent on TLR4/MyD88 downstream signaling. TNF-α is first released and signaling through its receptor triggers CXCL1 and IL-1β production (Calil et al., 2014). TNF-α and IL-1β also mediate pain signaling by directly sensitizing nociceptors (Binshtok et al., 2008; Jin and Gereau IV, 2006). Moreover, IL-1β activates neutrophils leading to production of PGE_2, a prostanoid shown to mediate mechanical and thermal hyperalgesia through PKA- and PKC-dependent mechanisms (Cunha et al., 2008; Kawabata, 2011). Increasing evidence has supported the role of IL-6 in pain signaling as well (Cunha et al., 1992; Zhou et al., 2016; Pinho-Ribeiro et al., 2017). The mechanisms underlying IL-6-induced nociception are not entirely described. For instance, IL-6 levels were shown to be increased in the spinal cord of rats following intraplantar injection of Complete Freund's Adjuvant (CFA) (Sun et al., 2012; Xu et al., 2014). IL-6 was also demonstrated to play a role in nociceptor sensitization as well as central sensitization supporting its participation on pathological pain (Brenn et al., 2007; Obreja et al., 2002; Vazquez et al., 2012). Therefore inhibition of TNF-α, IL-1β, and IL-6 are likely involved in the analgesic effect of probucol as well. These findings are in agreement with previous studies where probucol also reduced TNF-α, IL-1β and / or IL-6 levels after LPS challenge in murine microglial cells, human peripheral blood mononuclear cells and endothelial cells (Jung et al., 2016; Meng et al., 2004; Park et al., 2008). Importantly, probucol-mediated inhibition of cytokine production in the paw tissue, peritoneal

cavity and macrophage culture further supports its inhibitory effect on upstream signaling such as the NF-κB pathway.

Conclusion

Taken together our results indicate that probucol presents anti-inflammatory and analgesic activities by targeting the NF-κB signaling cascade. We propose that probucol acts on resident immune cells, such as macrophages, preventing the amplification and persistence of the inflammatory response, which depends on NF-κB-related cytokines. This in turn reduces neutrophil recruitment and induces analgesia (Fig. 8). Probucol could be a useful anti-inflammatory and analgesic together with antibiotic treatment. Therefore by controlling rather than abrogating the inflammatory response elicited by LPS, our results suggest that probucol is a putative candidate for the treatment of LPS-induced inflammation and hyperalgesia.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Figures

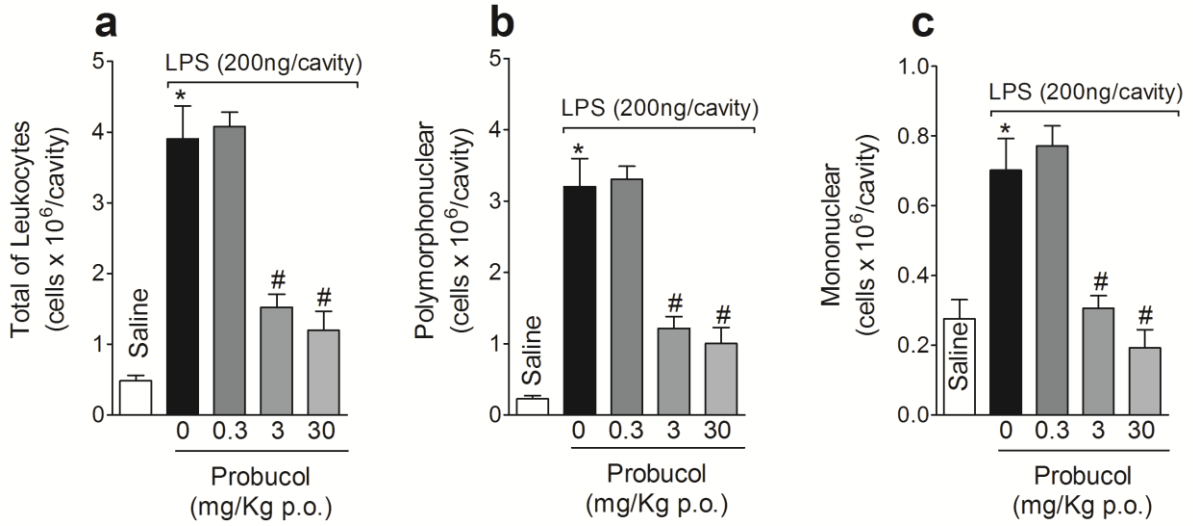

Fig. 1 Probucol inhibits LPS-induced leukocyte recruitment in the peritoneal cavity. Mice received probucol at doses of 0.3, 3 and 30 mg/kg (p.o.) 1 h before i.p. injection of LPS (200 ng/cavity). Total leukocytes (a) and differential counts of polymorphonuclear (b) and mononuclear (c) cells in peritoneal exudate were performed 6 h after stimulus. The 0 mg/kg of probucol group stands for vehicle group. Results are expressed as mean ± SEM (n = 6 per group per experiment, representative of two separate experiments). One-way ANOVA followed by Tukey's post-hoc. *$p<0.05$ vs. saline group. #$p<0.05$ vs. vehicle group.

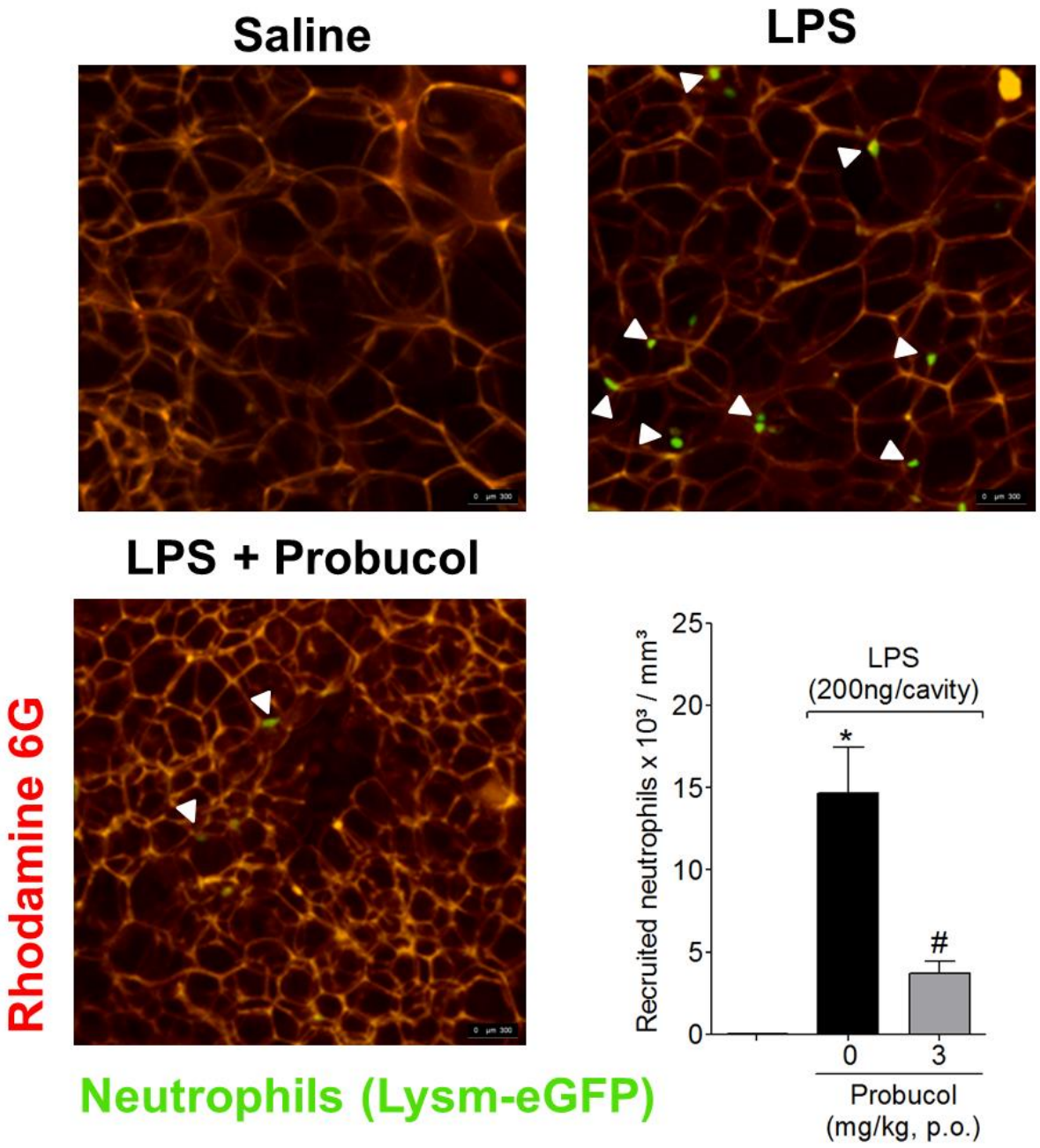

Fig. 2 Probucol inhibits LPS-induced neutrophil recruitment in the peritoneal cavity. Lysm-eGFP mice received probucol at 3 mg/kg (p.o.) 1 h before i.p. injection of LPS (200 ng/cavity). Neutrophil recruitment (arrowheads) was evaluated 4 h after LPS stimulus. The 0 mg/kg of probucol group stands for vehicle group. 20_x Objective. Scale bars, 300 μm. Results are expressed as mean ± SEM (n = 3 per group per experiment, representative of two separate experiments). One-way ANOVA followed by Tukey's post-hoc. *p<0.05 vs. saline group. #p<0.05 vs. vehicle group.

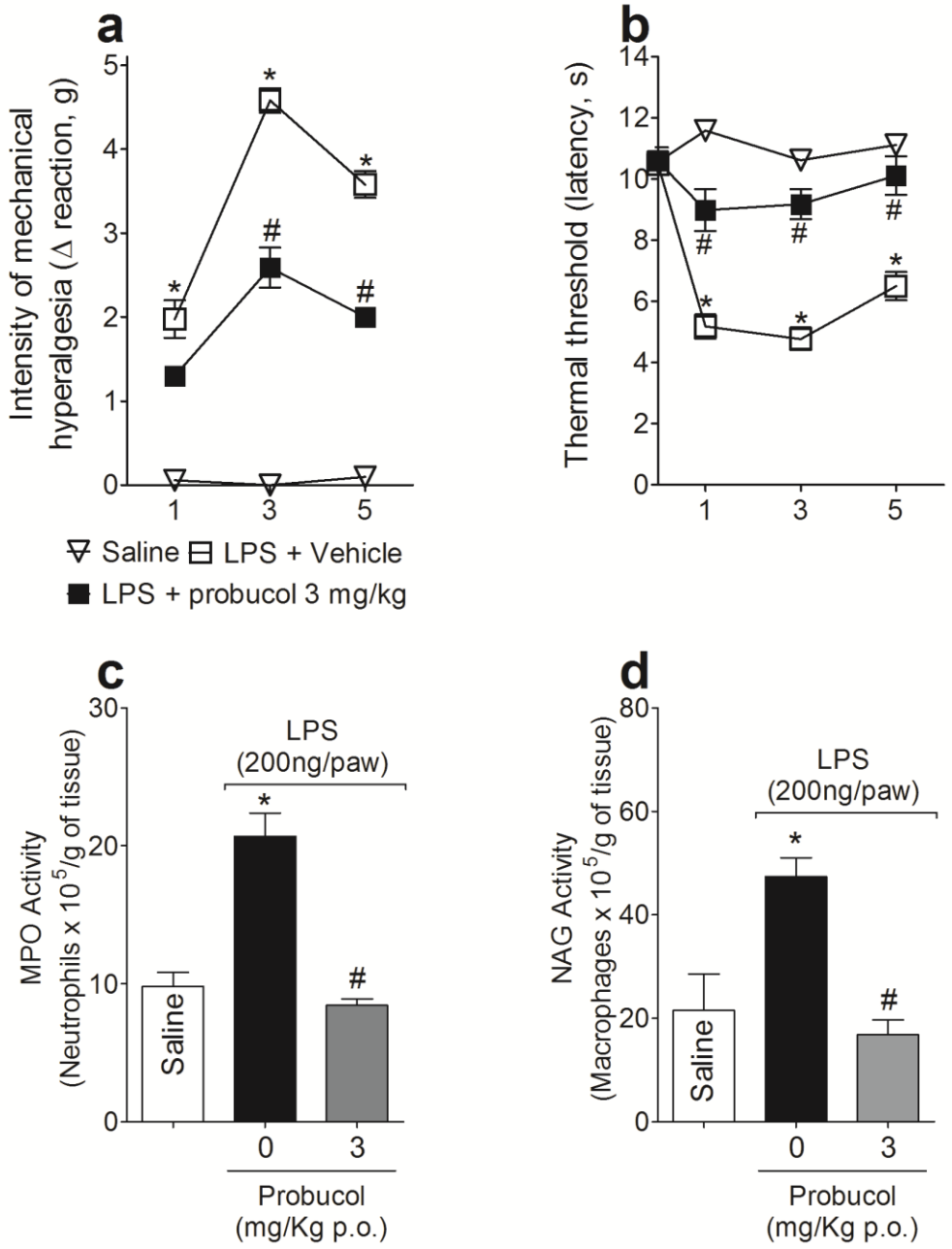

Fig. 3 Probucol inhibits LPS-induced mechanical and thermal hyperalgesia. Mice received probucol at 3 mg/kg (p.o.) 1 h before i.pl. injection of LPS (200 ng/paw). Evaluation of mechanical (a) and thermal (b) were performed 1, 3 and 5 h after stimulus. MPO (c) and NAG (d) activities were assessed 5 h after LPS injection. Results are expressed as mean ± SEM (n = 6 per group per experiment, representative of two separate experiments). Two-way repeated measures ANOVA followed by Tukey's post-hoc (a) and one-way ANOVA followed by Tukey's post-hoc (b). *$p<0.05$ vs. saline group. #$p<0.05$ vs. vehicle group.

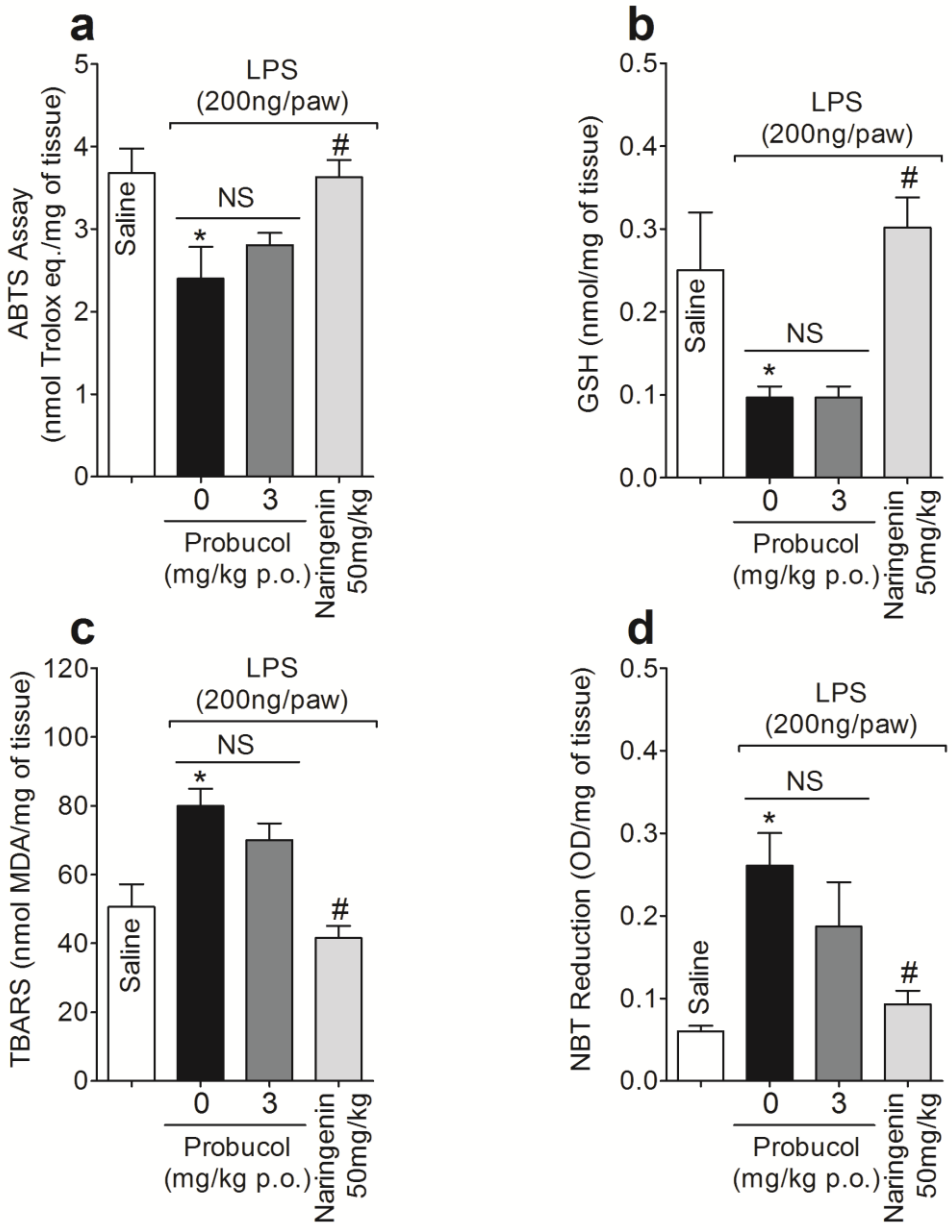

Fig. 4 Probucol does not affect LPS-induced oxidative stress in the paw tissue. Mice received probucol at 3 mg/kg (p.o.) or the antioxidant naringenin at 50 mg/kg (p.o.) 1 h before i.pl. injection of LPS (200 ng/paw). ABTS assay (a), GSH measurement (b), TBARS (c) and NBT (d) assays were performed from paw skin samples removed 3 h post LPS injection. The 0 mg/kg of probucol group stands for vehicle group. Results are expressed as mean ± SEM (n = 6 per group per experiment, representative of two separate experiments). One-way ANOVA followed by Tukey's post-hoc. *$p<0.05$ vs. saline group. #$p<0.05$ vs. vehicle group. NS stands for non-significant statistical differences.

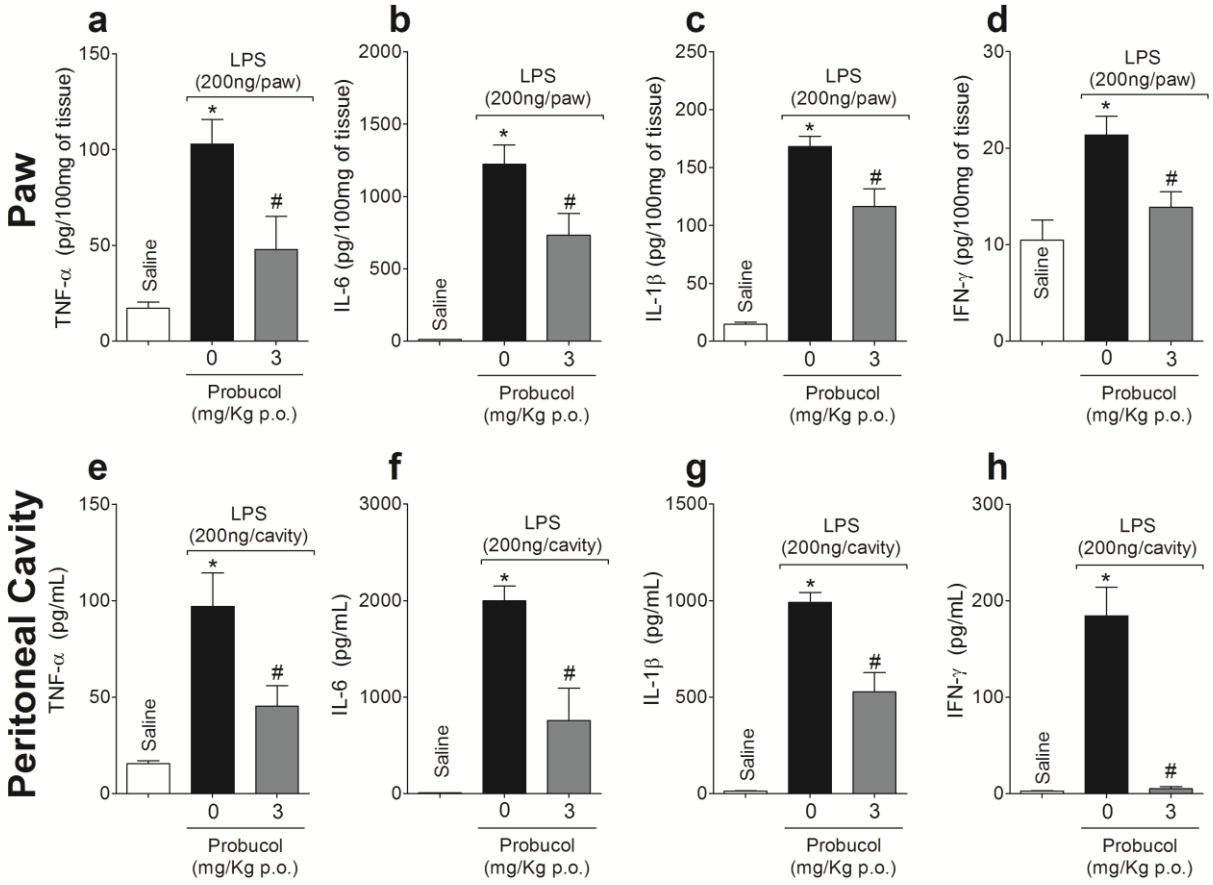

Fig. 5 Probucol inhibits LPS-induced cytokine production in paw tissue and peritoneal cavity. Mice received probucol at 3 mg/kg (p.o.) 1 h before i.pl. or i.p. injection of LPS (200 ng/paw). Paw skin was collected 3 h post injection and peritoneal exudate was collected 6 h post stimulus injection post LPS injection. TNF-α, IL-6, IL-1β and IFN-γ levels were measured by ELISA. The 0 mg/kg of probucol group stands for vehicle group. Results are expressed as mean ± SEM (n = 6 per group per experiment, representative of two separate experiments). One-way ANOVA followed by Tukey's post-hoc. *$p<0.05$ vs. saline group. #$p<0.05$ vs. vehicle group.

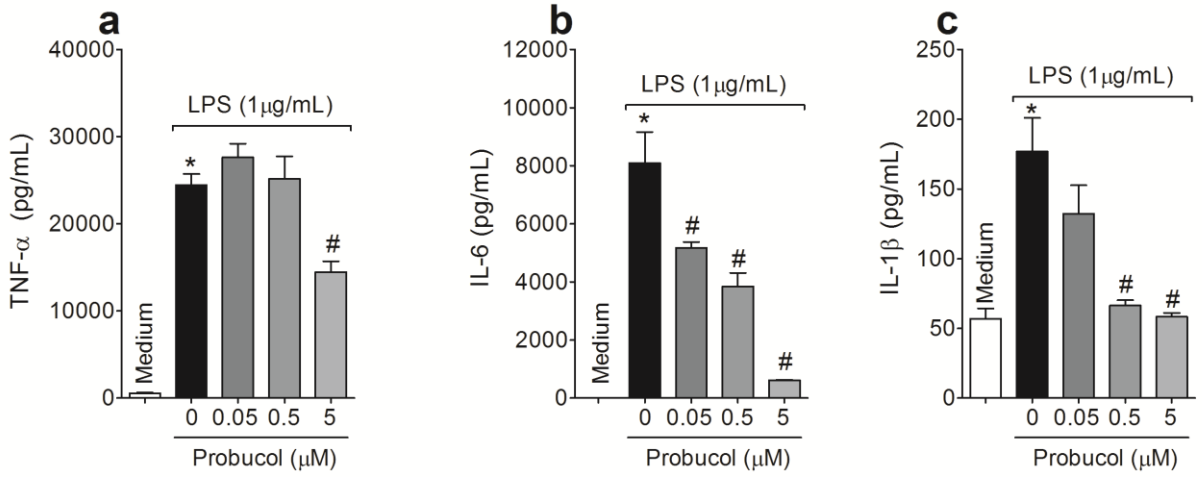

Fig. 6 Probucol inhibits LPS-induced cytokine production in macrophages. RAW 264.7 macrophages (3 x 10^5 cells per well) were treated with probucol (0.05-5 μM) or vehicle (Tween 80 5%) 1 h before LPS stimulus (1 μg/mL). Cytokine production was determined in supernatants 6 h after stimulus. Results are expressed as mean ± SEM (n = 5 per group per experiment, representative of two separate experiments). One-way ANOVA followed by Tukey's post-hoc. *p<0.05 vs. saline group. #p<0.05 vs. vehicle group.

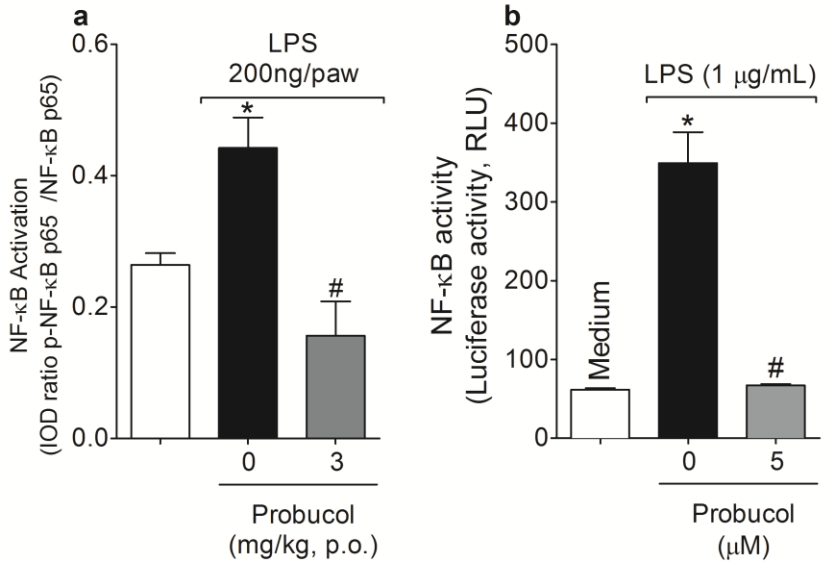

Fig. 7 Probucol inhibits LPS-induced NF-κB activation in paw and NF- κB activity in macrophages. Mice received probucol at 3 mg/kg (p.o.) 1 h before i.pl. injection of LPS (200 ng/paw). Paw skin was collected 3 h post injection. NF-κB activation was evaluated by ELISA. The 0 mg/kg of probucol group stands for vehicle group (a). RAW 264.7 macrophages (3 x 10^5 cells per well) were treated with probucol at 5 μM or vehicle (Tween 80 5%) 1 h before LPS stimulus (1 μg/mL). NF-κB activity was determined in cell lysates 6 h after stimulus (b). Results are expressed as mean ± SEM (n = 6 per group per experiment, representative of two separate experiments). One-way ANOVA followed by Tukey's post-hoc. *p<0.05 vs. saline group. #p<0.05 vs. vehicle group.

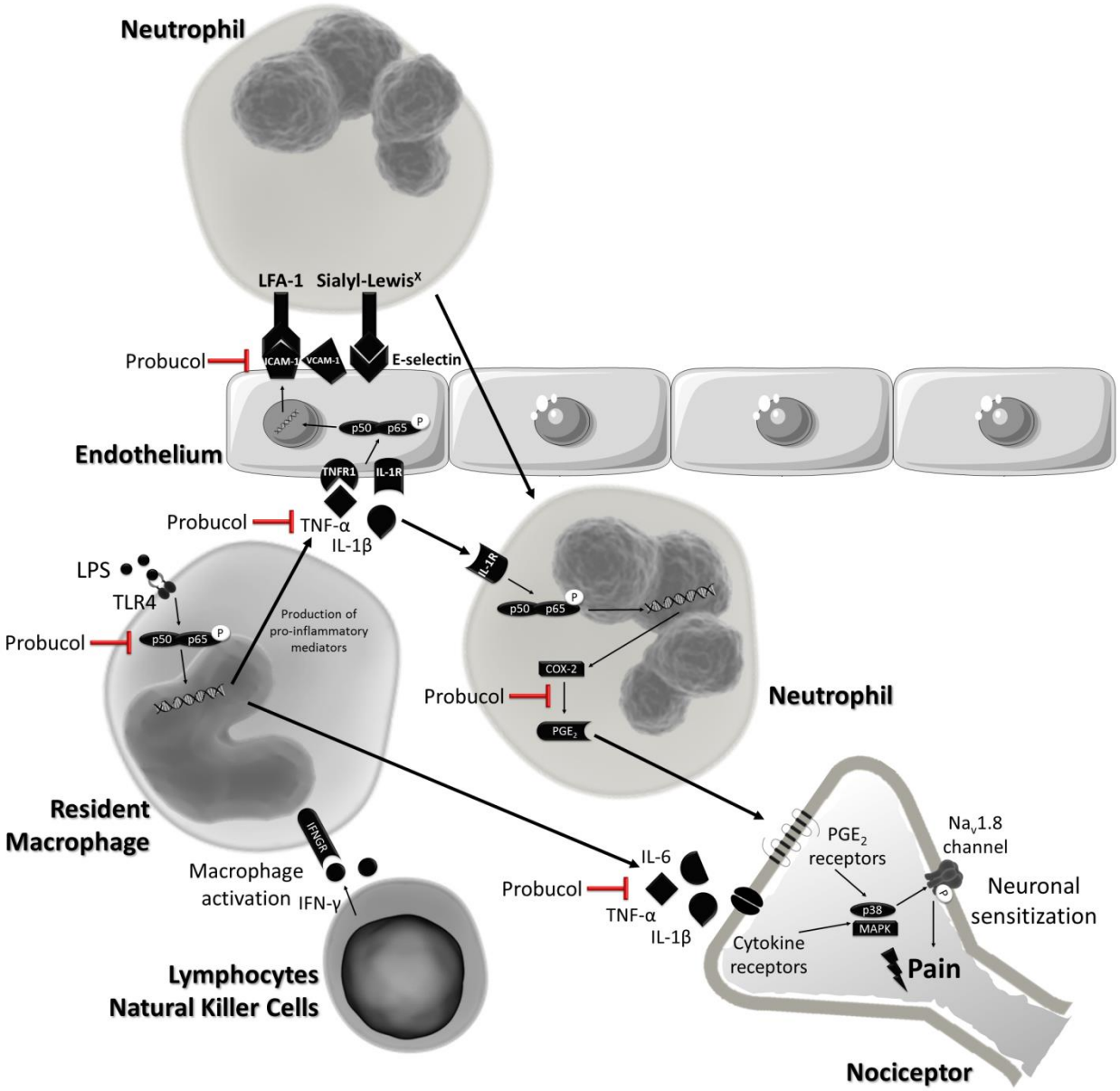

Fig. 8 Schematic representation of the proposed anti-inflammatory and analgesic mechanisms of probucol. Probucol acts on resident macrophages inhibiting LPS-driven NF-κB activation and cytokine production. As a result, there is reduction of endothelial cells activation and neutrophil recruitment. Neutrophils and macrophages play an important role in nociceptor sensitization by producing pro-hyperalgesic mediators such as PGE_2 and cytokines. Therefore, probucol impairs pain signaling causing analgesia by inhibiting macrophage activation and neutrophil recruitment. In support of these conclusions and present data, previous studies using experimental conditions different of the present study have shown probucol-mediated inhibition of NF-κB activation and well as production and / or expression of IL-1β, TNF-α, IL-6, PGE_2 and adhesion molecules ICAM-1, VCAM-1 and E-selectin (AKESON et al., 1991; AOKI et al., 2001; CHANG et al., 2010; CHEN et al., 2003; FERNS et al., 1993; KANEKO et al., 1996; LI et al., 2011; LIU et al., 2000; MENG et al., 2004; OZAKI et al., 1999; ZHANG et al., 2009; ZHANG et al., 2013; ZAPOLSKA-

DOWNAR et al., 2000). COX-2, cyclooxygenase 2. ICAM-1, Intercellular adhesion molecule 1. IFGNR, IFN-γ receptor. IL-1R, IL-1β receptor. LFA-1, Lymphocyte function-associated antigen 1. PGE_2, Prostaglandin E_2. TLR4, Toll-like Receptor 4. TNFR1, TNF-α receptor 1. VCAM-1, Vascular cellular adhesion molecule 1.

5 ARTIGO PARA PUBLICAÇÃO (INFLAMMATION RESEARCH)

O presente trabalho foi realizado na Universidade Estadual de Londrina, no Laboratório de Dor, Inflamação, Neuropatia e Câncer. Os resultados parciais estão descritos no artigo intitulado "Probucol ameliorates complete Freund adjuvant-induced inflammatory hyperalgesia by targeting the NF-κB pathway in paw and spinal cord", sob as normas da revista Inflammation Research.

Probucol ameliorates complete Freund adjuvant-induced inflammatory hyperalgesia by targeting the NF-κB pathway in paw and spinal cord

Amanda Z. Zucoloto[1]; Marília F. Manchope[1]; Victor Fattori[1]; Rubia Casagrande[2], Waldiceu A. Verri, Jr.[1]

[1]Departamento de Ciências Patológicas, Centro de Ciências Biológicas, Universidade Estadual de Londrina, Londrina, Brazil

[2]Departamento de Ciências Farmacêuticas, Centro de Ciências da Saúde, Universidade Estadual de Londrina, Londrina, Brazil

*** Correspondence:**

Dr. Waldiceu A. Verri Jr.

waldiceujr@yahoo.com.br; waverri@uel.br; + 55 43 3371 4979

Keywords: Subacute inflamation, Probucol, Pain, Nociception, CFA.

Abstract

Objective and design: This study aimed to evaluate the effect of probucol in inflammatory hyperalgesia and leukocyte recruitment in a model of subacute inflammation by CFA.

Treatment: Probucol at 0.3-3 mg/kg was administrated per oral daily 24 h after CFA intraplantar injection.

Methods: Mechanical and thermal hyperalgesia induced by CFA were determined using an electronic anesthesiometer and hot plate apparatus, respectively. Paw edema was measured using a dial thickness gauge. Overt pain-like behaviors were determined by the number of flinches and time spent licking paw, neutrophil recruitment by determination of myeloperoxidase activity, antioxidant effect by ABTS, NBT and TBARS assays, cytokine production and NF-κB activation by ELISA and *Iba-1*, *Gfap*, *Cox*-2 and *PreproET*-1 expression by qPCR. Data were analyzed by ANOVA followed by Tukey's post-hoc. $p<0.05$ was considered significant.

Results: Probucol reduced CFA-induced mechanical and thermal hyperalgesia, paw edema, overt pain-like behavior and myeloperoxidase activity in paw. Additionally, probucol reduced cytokine levels and NF-κB activation in paw skin and spinal cord as well as *Gfap*, *Cox*-2 and *PreproET-1* levels in the spinal cord.

Conclusion: Probucol presents analgesic and anti-inflammatory activities in experimental model of subacute inflammation. The results suggest its mechanisms of action seem to involve targeting of NF-κB.

Introduction

Pain is a cardinal feature of inflammation and an evolutionary mechanism of self-preservation. Nonetheless persistence of noxious stimuli and impairment in resolution processes underlie the pathogenesis of most chronic inflammatory diseases [1,2]. Pathological pain is a comorbidity of many chronic diseases and an emerging public health concern with social and economic implications. Therefore treating chronic pain is of great importance as it affects patient's quality of life and productivity [3–5]. The side effects associated with long-term use of steroidal or non-steroidal anti-inflammatory drugs challenge the treatment of chronic diseases [6,7]. In that sense development of alternative therapies is of great relevance worldwide. Experimental models of subacute inflammation and persistent pain have been useful to screen and characterize the analgesic and anti-inflammatory potential of novel compounds.

Complete Freund adjuvant (CFA) is a water-in-oil emulsion made of non-metabolizable oils and heat killed *Mycobacterium tuberculosis* (MTB). MTB cell wall structures are sensed by at least 3 different toll-like receptors (TLRs) among other PRRs, such as NOD2 and Dectin-1 [8–12]. MTB cell wall glycolipids and lipoproteins activate TLR2 leading to TNF-α and IL-1β production in macrophages [13,14]. Signaling through Dectin-1 and NOD2 also induces production of pro- and anti-inflammatory cytokines, e.g. TNF-α, IL-6 and IL-10 [10,11]. Moreover Means et al. (2001) demonstrated the crucial role of TLR2 and TLR4 in triggering the NF-κB pathway in macrophages stimulated with MTB [12].

CFA mediates long-lasting nociception characterized by glial activation and spinal production of pro-inflammatory mediators as early as 4 h culminating in thermal and mechanical hyperalgesia for up to 14 days post-injection. Many inflammatory mediators are increased upon CFA administration such as the pro-inflammatory cytokines IL-1β, TNF-α and IL-6 in both peripheral tissues as well as the spinal cord. The peptide endothelin was also shown to be involved in CFA-induced inflammation and hyperalgesia [15]. Therefore the CFA model is a well-established model to evaluate the analgesic mechanisms of novel compounds in all stages of the inflammatory response, i.e., acute, subacute and chronic.

Probucol is a synthetic polyphenolic compound firstly exploited as a lipid-lowering agent [16]. However the beneficial effects of probucol were shown to go beyond the lipid metabolism as its antioxidant and anti-inflammatory mechanisms started to be unveiled. For instance probucol acts as a superoxide anion scavenger *in vitro* [17]. It was also shown to inhibit production of pro-inflammatory and pro-hyperalgesic mediators as well as expression of adhesion molecules and NF-κB activation *in vitro* [18–23]. These mechanisms likely contribute to the beneficial effects of probucol observed in experimental models of atherosclerosis, neurodegenerative diseases and diabetes [24–27].

Given these previous studies we sought to evaluate the analgesic potential of probucol in the CFA model of persistent pain. To further investigate the mechanisms involved in its antinociceptive mechanisms we evaluated the production of pro-inflammatory and pro-hyperalgesic cytokines as well as NF-κB activation in peripheral tissue and in the spinal cord. We have also evaluated the effect of probucol on glial activation in the spinal cord.

Materials And Results

Animals

Male Swiss mice (20-25g) from Universidade Estadual de Londrina, Parana, Brazil were used in this study. Mice were housed in standard clear plastic cages, and received food and water *ad libitum* under a 12:12 h light/dark cycle at 21 °C. All behavior testing were performed between 9 am and 5 pm in temperature-controlled room. The Animal Welfare and Ethics Committee of Londrina State University approved this study (process number 1012.2015.74). All efforts were made to minimize the number of animals used and their suffering.

Drugs

Materials were obtained from the following sources: Complete Freund Adjuvant (Sigma-Aldrich, St. Louis, MO, USA), Diclofenac (Neoquímica, Anápolis, GO, Brazil), Indomethacin (Sigma-Aldrich, St. Louis, MO, USA), Acetaminophen (Santa Cruz Biotechnology, Dallas, TX, USA), Probucol (Santa Cruz Biotechnology, Dallas, TX, USA).

Experimental procedures

Mice were treated with probucol (0.3, 1, 3 mg/kg, per oral) or vehicle (Tween 80 5% in saline) daily 24 h after the administration of CFA (10 µL/paw). Mechanical hyperalgesia, thermal hyperalgesia and paw edema were evaluated 3 h after treatment for 7 days. Paw skin was removed to assess neutrophil recruitment by myeloperoxidase (MPO) activity assay 7 days after CFA injection. Antioxidant effect, TNF-α, IL-6, IL-1β and IL-10 levels and NF-κB activation were evaluated in paw and spinal cord (L4-L6) 3 days after CFA i.pl. injection as well as spinal expression of *Iba-1*, *Gfap*, *Cox*-2 and *PreproET*-1. Overt pain-like behavior was induced by CFA (10 µL/paw) and the number of flinches and time spent licking the paw were evaluated for 30 min. In these set of experiments particularly mice were treated with probucol 1 h before CFA stimulus. Doses of stimulus and time points of sample collection were based on previous studies [15,28,29].

Mechanical hyperalgesia test

Mechanical hyperalgesia was assessed by an electronic version of von Frey filaments [30]. A handheld force transducer (electronic anesthesiometer; Insight, Ribeirao Preto, SP, Brazil) adapted with a 0.5 mm^2 polypropylene tip was used to evoke hind paw nociceptive withdrawal response. The intensity of the pressure (in g) at the moment of paw withdrawal was automatically recorded. The mechanical threshold was tested before (baseline) and after CFA i.pl. administration (10 µL/paw). The results are expressed as delta (Δ) withdrawal threshold (in g), obtained by subtracting the measurements at each time point (1 to 7 days after i.pl. CFA injection) from the baseline values.

Thermal hyperalgesia test

Animals were place on a hot plate apparatus (Insight, Ribeirao Preto, SP, Brazil) at a constant temperature of 52 °C. The end-point was characterized by removal of the paw followed by flinching or licking the paw, and latency time until the end-point reaction was determined [31]. Maximum latency until end-point was set at 20 s to avoid tissue damage. The results are expressed as means of latency (s) within experimental groups.

Myeloperoxidase assay

MPO kinetic-colorimetric assay was performed as an indirect indicator of neutrophil recruitment to paw tissue, respectively. Paw skin samples were collected 7 days after CFA injection and homogenized in ice-cold K_2HPO_4 buffer (400 µL, 50 mM, pH 6.0) containing HTAB (0.5% weight/volume) using a tissue-tearor (Biospec, Bartlesville, OK, USA). Samples were centrifuged (16100 *g* x 2 min x 4 °C) and the supernatants were used. The MPO activity assay was performed as previously described [32]. Briefly, 50 mM phosphate buffer, pH 6.0

containing 0.167 mg/ mL o-dianisidine dihydrochloride and 0.015 % hydrogen were added to samples and the absorbance was read at 450nm (Multiskan GO Microplate Spectrophotometer, Thermo Scientific, Vantaa, Finland). The values obtained were compared to a standard curve of neutrophils and results are expressed as MPO activity (neutrophils x 10^6 / g of tissue).

Paw edema

Paw edema was measured using a dial thickness gauge 0-20 mm (Mitutoyo, Andover, Hampshire, UK). The results are expressed as paw thickness (in mm). The values were obtained by subtracting the baseline values from the measurements obtained at each time point (1 to 7 days after i.pl. CFA injection).

Total Antioxidant Capacity

Antioxidant capacity was assessed by the ABTS assay. This test was adapted to a 96-well microplate format as previously described [29]. Paw skin and spinal cord (L4-L6) samples were collected 3 days after stimulus (CFA 10 µL/paw) and homogenized with a tissue-tearor in ice-cold KCl buffer (500 µL, 1.15% w/v). Samples were centrifuged at 835 *g* at 4 °C for 10 min, and supernatants were used. ABTS assay was performed as an indicator of the sample's ability to scavenge the free radical ABTS. Diluted ABTS solution was added to samples and the absorbance was measured at 730 nm after 6 min of incubation at 25 °C (Multiskan GO, Thermo Scientific). The results were equated against a standard Trolox curve (0.02-20 nmol) and expressed as nmol Trolox eq. per mg of tissue.

Superoxide anion production

Superoxide anion production was assessed by the NBT assay. Paw skin and spinal cord (L4-L6) samples were collected 3 days after stimulus (CFA 10 µL/paw). Samples were homogenized with a tissue-tearor in 500 µL of ice-cold KCl buffer (1.15% w/v) and the homogenates were used for the assay. The test was adapted to a microplate as previously described [33]. The NBT reduction was measured at 600 nm (Multiskan GO, Thermo Scientific). The results are expressed as optical density (OD) per mg of tissue.

Lipid peroxidation

Lipid peroxidation was measured by the TBARS (Thiobarbituric Acid Reactive Substances) assay. Paw skin and spinal cord (L4-L6) samples were collected 3 days after stimulus (CFA 10 µL/paw) and homogenized with a tissue-tearor in ice-cold KCl buffer (500 µL, 1.15% w/v). The test was adapted to a microplate as previously described [33]. The intermediate product of lipid peroxidation malondialdehyde (MDA) was determined by subtracting the absorbance at 535 nm from the absorbance at 572 nm Multiskan GO, Thermo Scientific). The results are expressed as nmol of MDA per mg of tissue.

Cytokine measurement

Paw skin and spinal cord (L4-L6) samples were collected 3 days after stimulus (CFA 10 µL/paw). Samples were homogenized with a tissue-tearor in 500 µL of ice-cold buffer with protease inhibitors followed by centrifugation (835 *g* x 15 min x 4 °C). The supernatants of paw skin and spinal cord homogenates were used to determine TNF-α, IL-6, IL-1β and IL-10 levels by enzyme-linked immunosorbent assay (ELISA) using commercial kits (eBioscience, San Diego, CA, USA). Absorbance was measured at 450 nm (Multiskan GO, Thermo Scientific). The results are expressed as picograms of cytokines per 100mg of tissue.

NF-κB activation

Paw skin and spinal cord (L4-L6) samples were collected 3 days after stimulus (CFA 10 µL/paw). Samples were homogenized with a tissue-tearor in 400 µL of ice-cold lysis buffer (Cell Signaling, Danvers, MA, USA) followed by centrifugation (16100 *g* x 10 min x 4 °C). The supernatants were used for determination of phosphorylated NF-κB p65 subunit and total NF-κB p65 subunit levels using ELISA PathScan Kits (Cell Signaling, Danvers, MA, USA) according to the manufacturer's directions. Absorbance was measured at 450 nm (Multiskan GO, Thermo Scientific). The results are expressed as IOD ratio (p-NF-κB p65/total NF-κB p65).

2.13 Reverse-Transcriptase and Quantitative PCR

Spinal cord samples were collected 3 days after stimulus (CFA 10 µL/paw). Samples were homogenized in TRIzol® reagent (Life Technologies), and total mRNA was isolated according to manufacturer's directions. RNA purity was confirmed by the 260/280 ratio. Reverse-Transcriptase PCR (RT-PCR) was performed as previously described [33]. Quantitative PCR (qPCR) was performed using GoTaq® 2-Step RTqPCR System (Promega) on a StepOnePlus™ Real-Time PCR System (Applied Biosystems®). The relative gene expression was measured using the comparative $2^{-(\Delta\Delta Cq)}$ method. The expression of GAPDH (glyceraldehyde 3-phosphate dehydrogenase) mRNA was used as a reference gene to normalize data. The primers used were *Gapdh*, sense: 5'CATACCAGGAAATGAGCTTG 3', anti-sense: 5' ATGACATCAAGAAGGTGGTG 3'; *Gfap*, sense: 5' GGCGCTCAATGCTGGCTTCA 3', anti-sense: 5' TCTGCCTCCAGCCTCAGGTT 3'; *Iba-1*, sense: 5' ATGGAGTTTGATCTGAATGGAAAT 3' , anti-sense: 5' TCAGGGCAGCTCGGAGATAGCTTT 3'; *Cox-2*, sense: 5´GTGGAAAAACCTCGTCCAGA 3´, anti-sense: 5´GCTCGGCTTCCAGTATTGAG 3´; *PropreET-1*, sense: 5' TGTGTCTACTTCTGCCACCT 3', anti-sense:. 5' CACCAGCTGCTGATAGATAC 3'.

Parameters of drug toxicity

To evaluate hepatotoxicity, plasma levels of aspartate aminotransferase (AST) e alanine aminotransferase (ALT) were measured using a diagnostic kit from Labtest (Lagoa Santa, Minas Gerais, Brazil) [34,35]. Plasmatic AST and ALT levels in probucol-treated mice were compared to acetaminophen treatment (650 mg/kg, single dose), a hepatotoxic drug. The results are expressed as units per L. In turn nephrotoxicity was assessed by measurement of urea and creatinine in plasma also using a diagnostic kit from Labtest (Lagoa Santa, Minas Gerais, Brazil) [36]. The results are expressed as mg per mL and were compared to diclofenac treatment (200 mg/kg, single dose). Gastric lesion was evaluated by performing the MPO kinetic-colorimetric assay as an indirect indicator of neutrophil recruitment in stomach [28]. The results are expressed as neutrophils per g of tissue and were compared to indomethacin treatment.

Statistical analyses

Results are presented as mean ± SEM of measurements made on 6 mice per group per experiment, and are representative of two independent experiments. Two-way repeated measures analysis of variance (ANOVA) followed by Tukey's post hoc were used to compare groups and doses at all time points when responses were measured at different time points after stimulus injection (mechanical and thermal hyperalgesia tests and edema assessment). One-way ANOVA followed by Tukey's post-hoc was performed for data from single time point experiments. $P < 0.05$ was considered statistically significant. All data analyses were performed using GraphPad Prism® 5.0 (GraphPad Software, Inc., USA-500.288), as well as elaboration of figures.

Results

Probucol inhibits CFA-induced mechanical and thermal hyperalgesia, neutrophil recruitment and paw edema

Mice were treated with probucol at doses 0.3, 1 and 3 mg/kg p.o. or vehicle (Tween 80 5% in saline) daily 24 h after i.pl. administration of CFA (10 µL/paw). At day 1, mechanical and thermal hyperalgesia were evaluated 1, 3, 5 and 7 h after treatment. The analgesic effect was more prominent 3 h after treatment so this time was chosen to evaluate both mechanical and thermal hyperalgesia the following days (data not shown). Probucol at 3 mg/kg reduced CFA-induced mechanical hyperalgesia at all time points, up to 45% (Fig. 1a). Thermal hyperalgesia was also reduced at all time points (up to 98%) (Fig. 1b). Probucol at doses 0.3 mg/kg or 1 mg/kg did not show analgesic effect ($p > 0.05$). Paw tissue was removed 7 days after CFA stimulus and the samples were used to evaluate MPO activity, a marker of neutrophil recruitment. Probucol at 3 mg/kg reduced MPO activity in paw (65%) similarly to the reference drug indomethacin, which showed a 62% MPO activity inhibition. In turn the lower doses of probucol did not affect neutrophil recruitment ($p > 0.05$) (Fig. 1c). Therefore the dose of 3 mg/kg was chosen for the next experiments. Paw edema was evaluated over the course of 7 days as well. A significate reduction of paw edema by probucol was only observed at days 1, 4 and 5 (up to 26%, at day 5). In turn indomethacin inhibited edema in all time points ($p < 0.05$) (Fig. 1d).

Probucol inhibits CFA-induced overt pain-like behavior

Mice were treated with probucol at 3 mg/kg (p.o.) 1 h before i.pl. injection of CFA (10 µL/paw). Overt pain-like behavior was evaluated over 30 minutes. Probucol inhibited CFA-induced 43% of paw flinches and 42% of time spent licking the paw (Fig. 2).

Probucol does not inhibit CFA-induced ROS production in paw and spinal cord

Mice were treated with probucol at 3 mg/kg p.o. or vehicle (Tween 80 5% in saline) daily 24 h after i.pl. administration of CFA (10 µL/paw). Paw skin and spinal cord tissue were 3 days after CFA i.pl. injection to evaluate the following parameters of oxidative stress: ABTS free radical scavenging ability, superoxide anion formation by NBT reduction and lipid peroxidation by TBARS. CFA induced tissue oxidative stress in paw and spinal cord observed as reduction of ABTS scavenging ability (22 and 40%, respectively) as well as superoxide anion formation (NBT reduction, 80 and 167%, respectively) and increase of lipid peroxidation (TBARS assay, 500 and 63%, respectively) and compared to the saline group. (Fig. 3a-f). Probucol at 3 mg/kg neither restored tissue antioxidant capacity nor reduced ROS formation ($p > 0.05$) (Fig. 4a-f).

Probucol inhibits CFA-induced cytokine production in paw and spinal cord

Mice were treated with probucol at 3 mg/kg p.o. or vehicle (Tween 80 5% in saline) daily 24 h after i.pl. administration of CFA (10 µL/paw). Paw skin and spinal cord tissue (L4-L6) were removed 3 days after CFA i.pl. injection. Probucol inhibited TNF-α (28% and 58%), IL-6 (56% and 58%), IL-1β (28% and 42%) and IL-10 (51% and 50%) production in paw and spinal cord, respectively (Fig. 4a-h).

Probucol inhibits CFA-induced NF-κB activation in paw and spinal cord

Mice were treated with probucol at 3 mg/kg p.o. or vehicle (Tween 80 5% in saline) daily 24 h after i.pl. administration of CFA (10 µL/paw). Paw skin and spinal cord tissue (L4-L6) were removed 3 days after CFA i.pl. injection. Probucol inhibited CFA-induced NF-κB activation in paw tissue (36%) and spinal cord (29%) demonstrated by a reduction of CFA-induced increase of p-NF-κB p65/NF-κB p65 ratio (Fig. 5a and b).

Probucol inhibits CFA-induced astrocytes activation and spinal expression of *Cox-2* and *PreproET-1*

Mice were treated with probucol at 3 mg/kg p.o. or vehicle (Tween 80 5% in saline) daily 24 h after i.pl. administration of CFA (10 µL/paw). Spinal cord tissue (L4-L6) was removed 3 days after CFA i.pl. injection. CFA increased expression of *Gfap* and *Iba-1* mRNA levels, markers of astrocyte and microglia activation, respectively. Treatment with probucol reduced *Gfap* mRNA levels whereas *Iba-1* levels remained unaffected (Fig. 6a and 6b, respectively). In turn probucol reduced spinal of both *Cox-2* and *PreproET-1* mRNA expression (Figures 6c and 6d, respectively).

Probucol treatment does not alter parameters of gastric lesions, renal and liver function

Mice were treated with probucol at 3 mg/kg p.o. or vehicle (Tween 80 5% in saline) for 7 days. Plasmatic levels of AST, ALT, creatinine and urea were evaluated using commercial diagnostic kits as markers of hepatotoxicity and nephrotoxicity, respectively. Neutrophil recruitment in stomach was assessed by MPO assay as a marker of gastric lesion. Treatment with probucol did not alter any of the parameters evaluated when compared to the vehicle-treated group ($p > 0.05$). Acetaminophen, diclofenac and indomethacin induced hepatotoxicity, nephrotoxicity and gastric lesion, respectively ($p < 0.05$) (Fig. 4).

Discussion

Up to 30% of adults suffer from chronic pain worldwide [5]. Persistent pain often becomes a limiting factor in most aspects of patients' lives, characterizing a relevant public health issue. Additionally, long-term treatment with currently used anti-inflammatory drugs presents a wide range of side effects also impairing patients' quality of life [6,7]. In this context, we aimed to investigate the analgesic and anti-inflammatory potential of probucol.

In the present work we have shown that probucol inhibited CFA-induced thermal and mechanical hyperalgesia along with overt pain-like behavior. Paw edema and neutrophil recruitment were also inhibited by probucol treatment. In the inflammatory milieu, neutrophils secrete PGE_2 in response to IL-1β. In turn PGE_2 directly sensitizes nociceptors triggering hyperalgesia [37]. Therefore inhibition of neutrophil influx and IL-1β production in paw may contribute to the analgesic effects observed.

Additionally, CFA components interact with a wide range of pattern recognition receptors (PRRs) eliciting an inflammatory response that persists for weeks. Previous studies have shown that CFA induces spinal activation provoking mechanical and thermal hyperalgesia in a NF-κB-dependent manner [15,38]. At day 4 post CFA, Zhu et al. (2014) observed mechanical and thermal hyperalgesia along with increased levels of TNF-α, IL-1β and IL-6 in lumbar spinal cord. Additionally, mRNA expression and protein levels of Iba-1, a marker of microglia activation, were increased at the same time point [39]. TNF-α, IL-1β and IL-6 were previously shown to be augmented in the spinal cord of rats as early as 4 hours post-CFA [15,40,41]. Since no systemic levels of cytokines were detected earlier than 24 h after CFA and the blood-brain barrier permeability was not increased up to 5 days post stimulus, it is likely that these pro-inflammatory cytokines are of central origin [42,43]. In fact, microglia activation was observed as early as 4 h after CFA and maintained up to 14 days. In turn astrocytes showed a delayed activation, at day 4 [15]. Hartung et al. (2015) demonstrated a role of astrocyte-derived NF-κB in inhibiting expression of catechol-o-methyltransferase, an enzyme that inactivates catecholamines involved in nociception [38].

In agreement with previous studies, we have observed increased mRNA expression of *Iba-1* and *Gfap*, i.e. activation of microglia and astrocytes, in the lumbar spinal cord as well as increased levels of IL-1β, TNF-α and IL-6. These mediators were shown to play an important role in pain signaling by either direct sensitization of nociceptors or by triggering production of other hyperalgesic mediators [44–47]. Probucol-treated mice showed reduced peripheral and spinal levels of IL-1β, TNF-α and IL-6 as well as reduced mRNA expression of *Gfap* suggesting inhibition of astrocyte activation and cytokine production are involved in its analgesic mechanisms.

In response to CFA, chemokines also play a role in neuronal sensitization. For instance, CXCL13 plays a role in CFA-induced nociception. CXCL13 of peripheral origin interacts with CXCR5 in DRG neurons activating p38. In turn p38 increases neuronal excitability through modulation of $Na_v1.8$ channels [48]. In the spinal cord, astrocytes produce CXCL1 which binds to CXCR2 in neurons driving expression of *Cox-2* [49]. Furthermore CXCL1 and CXCR2 were also shown to be upregulated in DRG neurons following CFA peripheral injection [50]. In accordance to this, we have shown a significant decrease in *Cox-2* mRNA expression in the lumbar spinal cord of probucol-treated mice 3 days after CFA. Transcription of *Cox*-2 and chemokines such as *Cxcl1* and *Cxcr2* is triggered upon NF-κB activation indicating probucol could target this upstream cascade [51–53].

We have also evaluated the effect of probucol on the spinal mRNA expression of *PreproET-1*, the precursor of endothelin 1 (ET-1). Endothelins are a family of peptides that signal through the G protein-coupled receptors ET_A and ET_B [54]. There is increasing evidence on the role of endothelins in pain signaling, including in CFA-induced inflammation [55,56]. ET-1 was shown to directly activate nociceptors as well as induce production of PGE_2 [57–59]. Additionally, *PreproET-1* is also a downstream target of the NF-κB pathway [51]. In the present work we have shown reduced levels of *PreproET-1* mRNA expression in the lumbar spinal cord of mice following treatment with probucol. This data further contributes to the growing range of pro-hyperalgesic mediators inhibited by probucol.

In order to investigate putative anti-inflammatory mechanisms induced by probucol we have evaluated antioxidant ability as well as IL-10 production in both spinal cord and paw tissue. Despite previous evidence, we have not observed an antioxidant effect at the anti-inflammatory/analgesic dose chosen in this work [24,60,61]. Our contrasting result can be explained by the use of a remarkably lower dose administrated for a short period (3 days) in opposed to longer treatments with higher doses, up to 61 mg/kg/day [24]. Therefore our results suggest probucol exerts anti-inflammatory and analgesic effects by employing mechanism other than its antioxidant properties. In regards to IL-10 levels, probucol inhibited its production in spinal cord as well as paw tissue suggesting it acts on an upstream target, possibly NF-κB itself. In fact, probucol has been previously shown to reduce NF-κB nuclear translocation in endothelial cells [18,23,62].

The canonical NF-κB pathway is activated upon stimulation of PRRs, IL-1R and TNFR1. Activated NF-κB translocates to nucleus triggering transcription of adhesion molecules and pro-inflammatory cytokines (e.g. IL-1β, TNF-α and IL-6). Moreover the NF-κB pathway displays a wide range of self-modulatory mechanisms. For instance, NF-κB promotes delayed transcription of anti-inflammatory mediators such as IL-10 [63,64]. Indeed, by assessment of the p-NF-κB p65/total NF-κB p65 ratio in both paw and lumbar spinal cord we have demonstrated the inhibitory effect of probucol on NF-κB activation.

Taken together our results strongly support that probucol alleviates persistent pain by inhibiting spinal and peripheral pro-hyperalgesic mediators in a NF-κB-dependent manner. Importantly daily treatment with probucol did not affect

renal or liver functions or caused gastric lesions, a common side effect of NSAIDs [7]. Thus our results broaden the clinical potential of probucol as a promising anti-inflammatory and analgesic drug.

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Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Figures

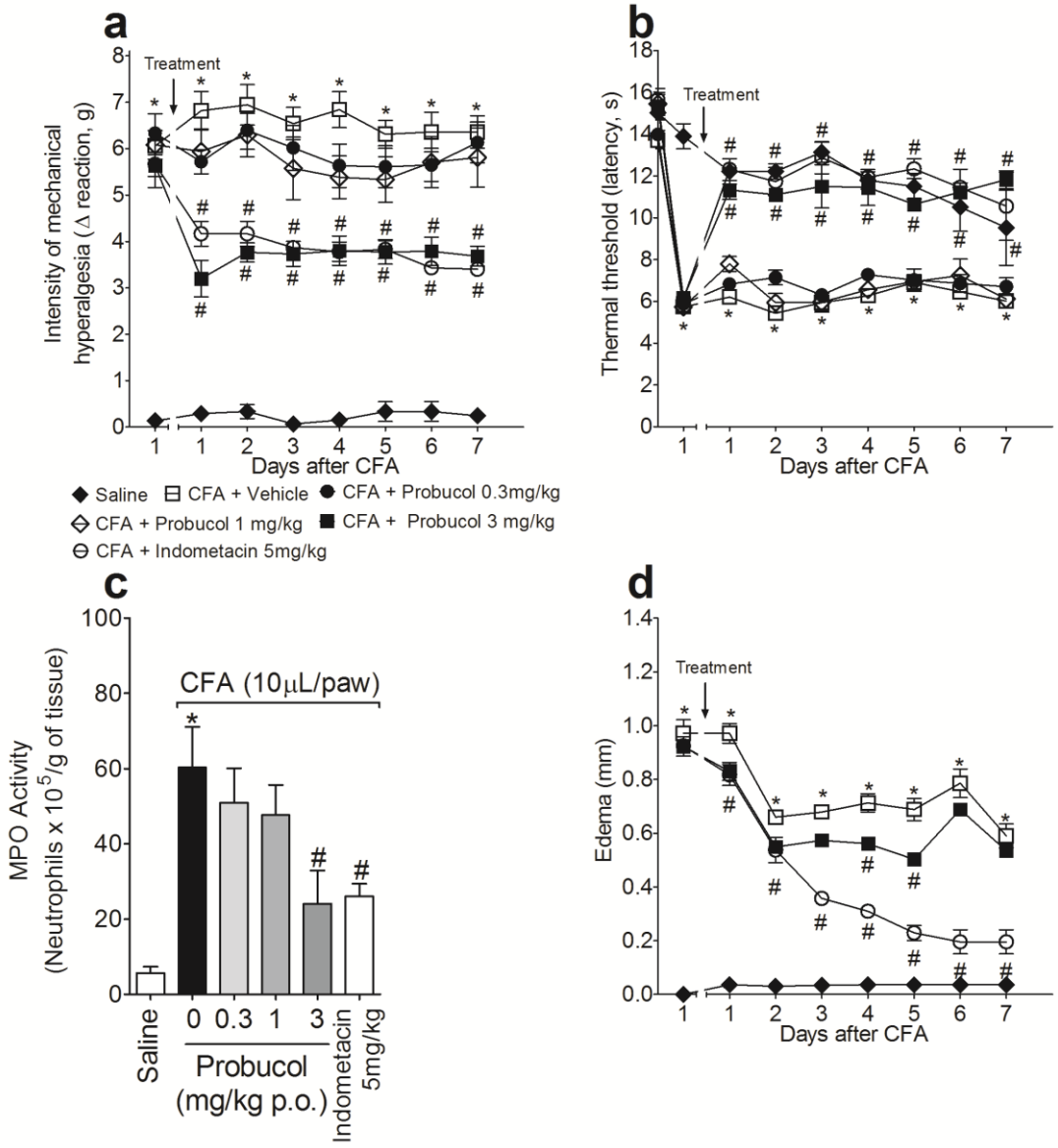


Fig. 1 Probucol inhibits CFA-induced mechanical and thermal hyperalgesia, neutrophil recruitment and paw edema. Mice received probucol at doses 0.3, 1 and 3 mg/kg (p.o.) daily 24 h after i.pl. injection of CFA (10 µL/paw). Mechanical (a) and thermal (b) hyperalgesia and paw edema (d) were evaluated 3 h after treatment for 7 days. MPO (c) activity was assessed 7 days after CFA stimulus. Results are expressed as mean ± SEM (n = 6 per group per

experiment, representative of two separate experiments). Two-way repeated measures ANOVA or One-way ANOVA followed by Tukey's post-hoc. *p<0.05 vs. saline group. #p<0.05 vs. vehicle group.

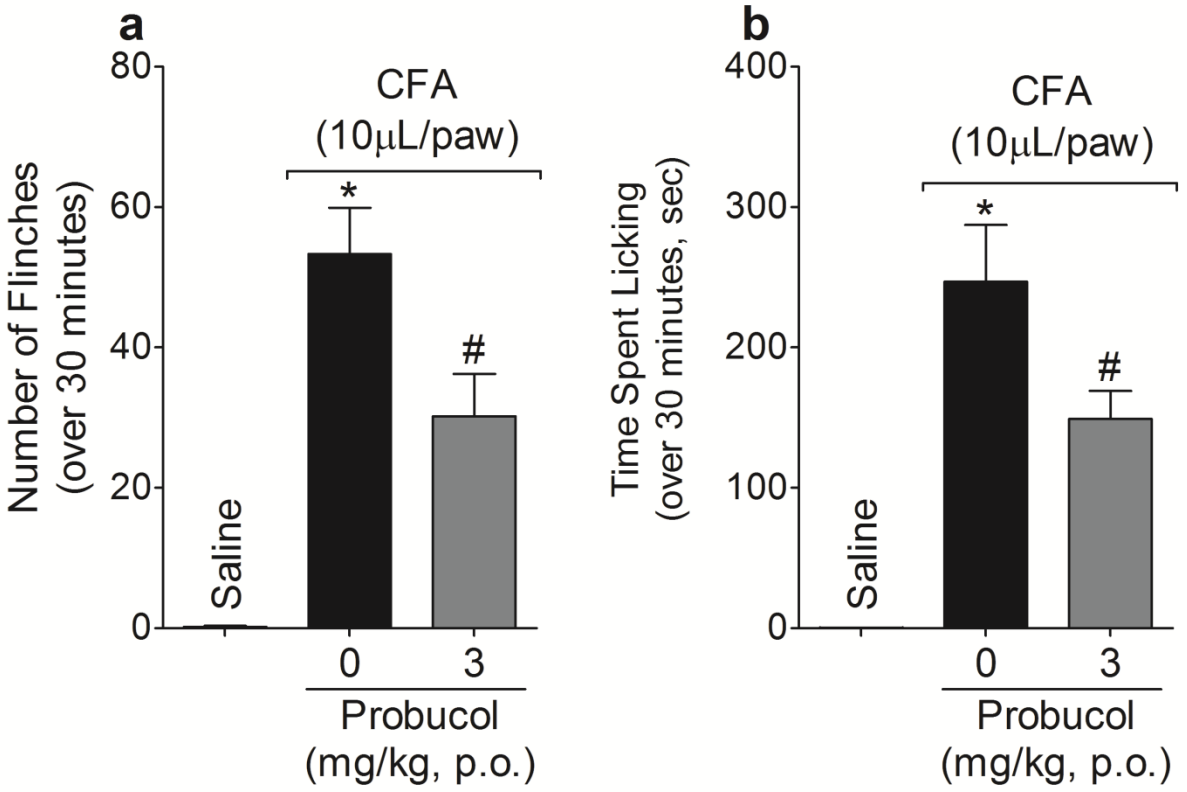


Fig. 2 Probucol inhibits CFA-induced overt pain-like behavior. Mice received probucol at 3 mg/kg (p.o.) 1 h before i.pl. injection of CFA (10 μL/paw). The number of flinches (a) and time spent licking the paw (b) were evaluated 0-30 min after i.pl. administration of CFA. Results are expressed as mean ± SEM (n = 6 per group per experiment, representative of two separate experiments). One-way ANOVA followed by Tukey's post-hoc. *p<0.05 vs. saline group. #p<0.05 vs. vehicle group.

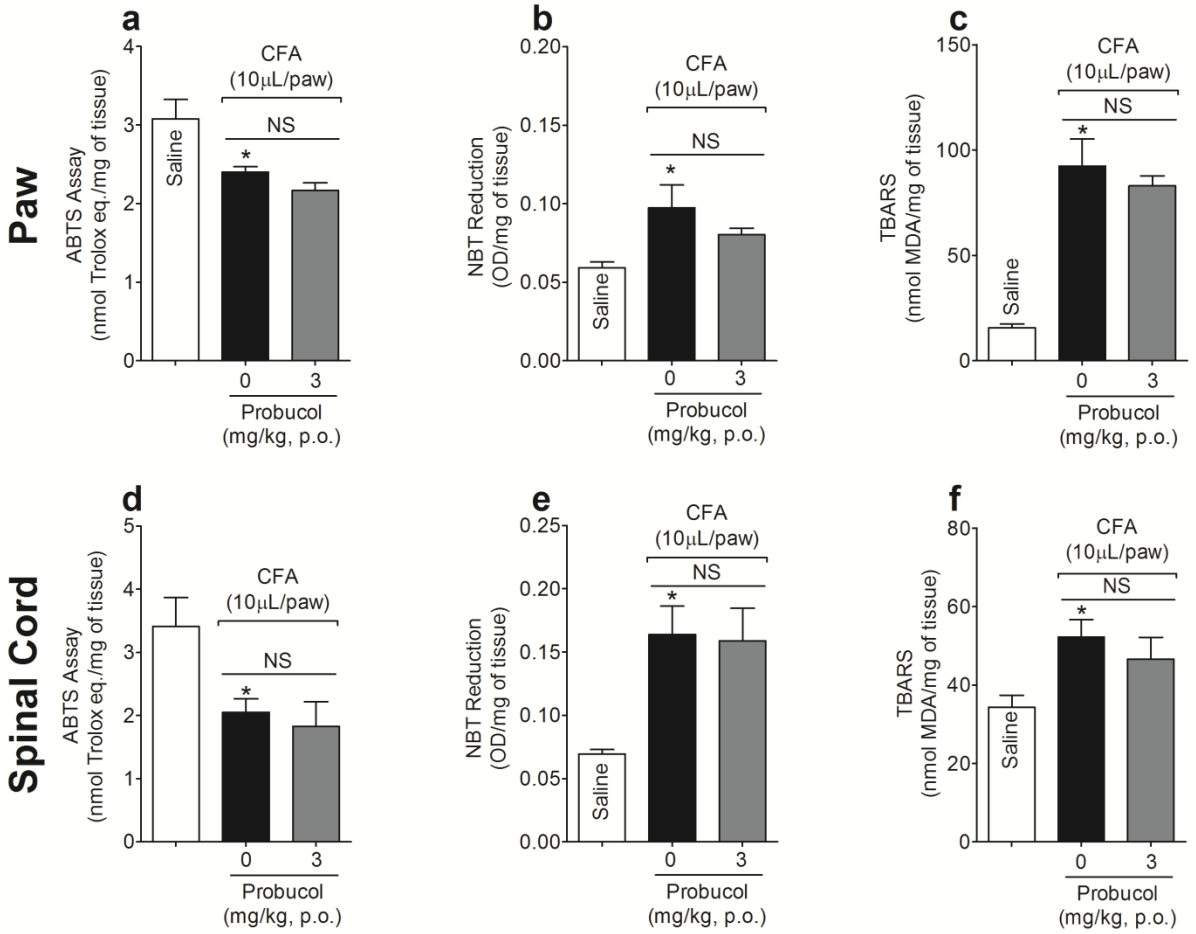


Fig. 3 Probucol does not inhibited CFA-induced ROS production in paw tissue and spinal cord. Mice received probucol at 3 mg/kg (p.o.) daily 24 h after i.pl. injection of CFA (10 µL/paw). Paw skin and spinal cord tissue (L4–L6) were removed 3 days after CFA. ABTS (a and d), NBT (b and e) and TBARS (c and f) assays were performed from paw skin and spinal cords. The 0 mg/kg of probucol group stands for vehicle group. Results are expressed as mean ± SEM (n = 6 per group per experiment, representative of two separate experiments). One-way ANOVA followed by Tukey's post-hoc. *p<0.05 vs. saline group. #p<0.05 vs. vehicle group. NS stands for non-significant statistical differences.

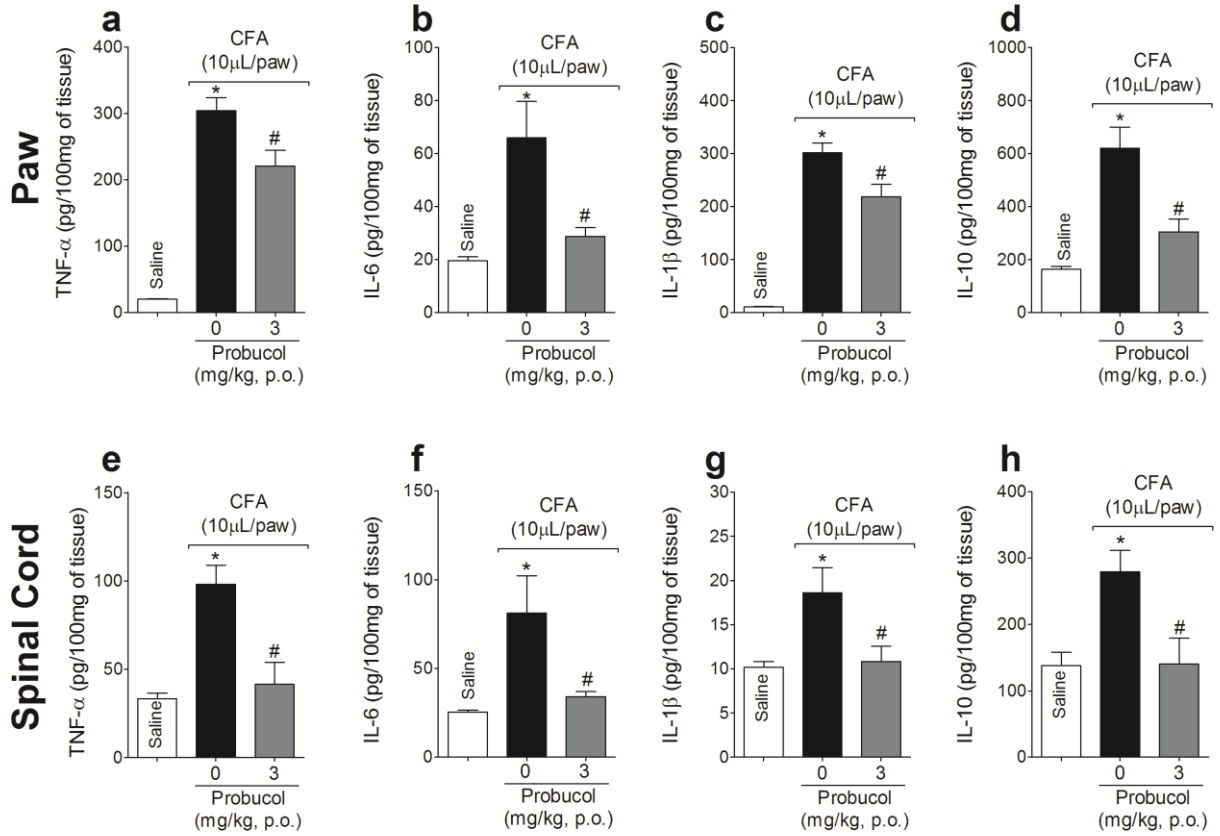


Fig. 4 Probucol inhibits CFA-induced cytokine secretion in paw tissue and spinal cord. Mice received probucol at 3 mg/kg (p.o.) daily 24 h after i.pl. injection of CFA (10 μL/paw). Paw skin and spinal cord tissue (L4–L6) were removed 3 days after CFA. TNF-α, IL-6, IL-1β and IL-10 levels were measured by ELISA. The 0 mg/kg of probucol group stands for vehicle group. Results are expressed as mean ± SEM (n = 6 per group per experiment, representative of two separate experiments). One-way ANOVA followed by Tukey's post-hoc. *$p<0.05$ vs. saline group. #$p<0.05$ vs. vehicle group.

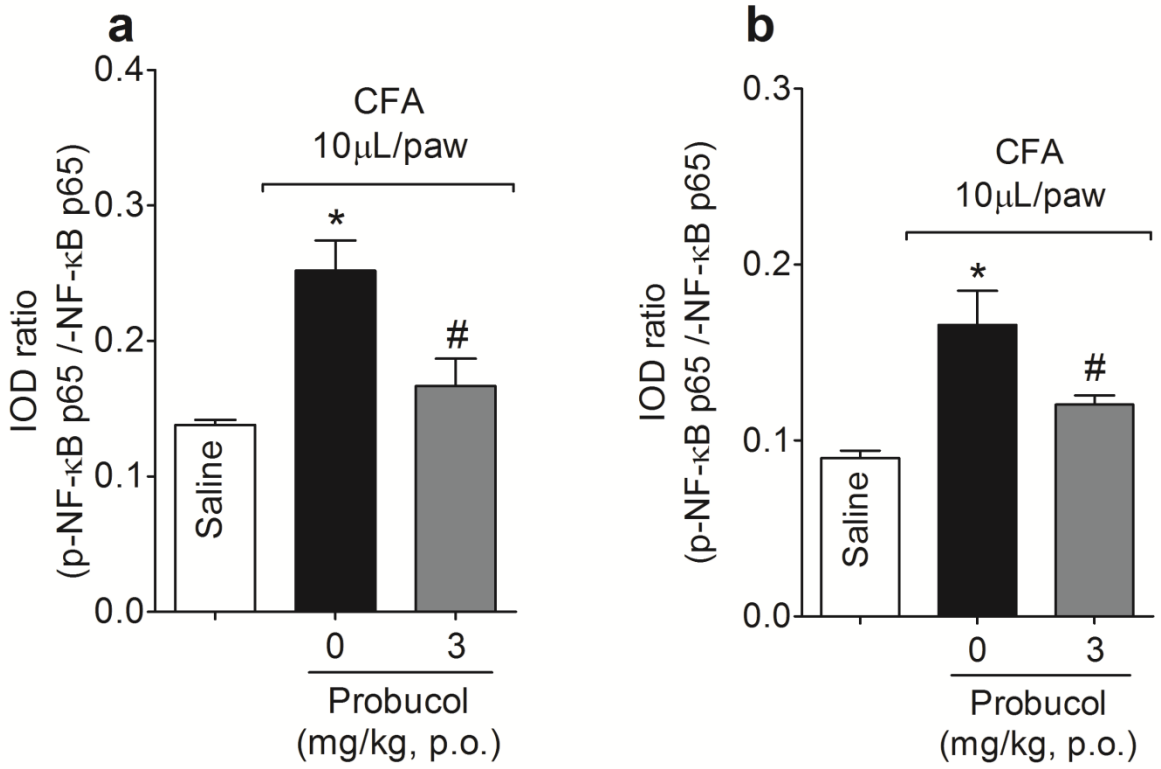


Fig. 5 Probucol inhibits CFA-induced NF-κB activation in paw tissue (a) and spinal cord (b). Mice received probucol at 3 mg/kg (p.o.) daily 24 h after i.pl. injection of CFA (10 μL/paw). Paw skin and spinal cord tissue (L4–L6) were removed 3 days after CFA. NF-κB activation was evaluated by ELISA. The 0 mg/kg of probucol group stands for vehicle group. Results are expressed as mean ± SEM (n = 6 per group per experiment, representative of two separate experiments). One-way ANOVA followed by Tukey's post-hoc. *$p<0.05$ vs. saline group. #$p<0.05$ vs. vehicle group.

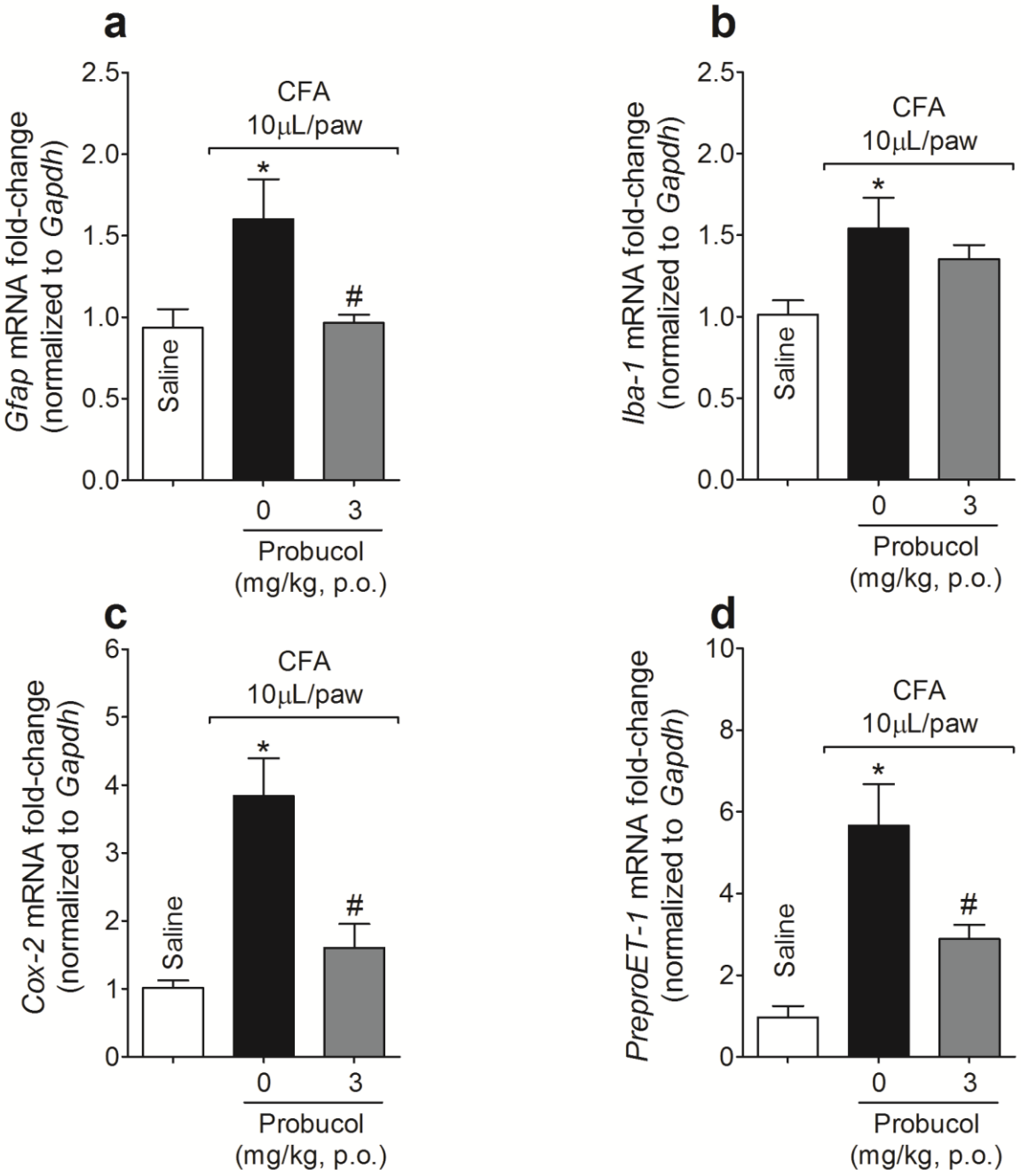


Fig. 6 Probucol inhibits CFA-induced expression of *Gfap*, *Cox-2* and *PreproET-1*. Mice received probucol at 3 mg/kg (p.o.) daily 24 h after i.pl. injection of CFA (10 μL/paw). Spinal cord tissue (L4–L6) was removed 3 days after CFA. NF-κB activation was evaluated by ELISA. The 0 mg/kg of probucol group stands for vehicle group. Results are expressed as mean ± SEM (n = 6 per group per experiment, representative of two separate experiments). One-way ANOVA followed by Tukey's post-hoc. *$p<0.05$ vs. saline group. #$p<0.05$ vs. vehicle group.

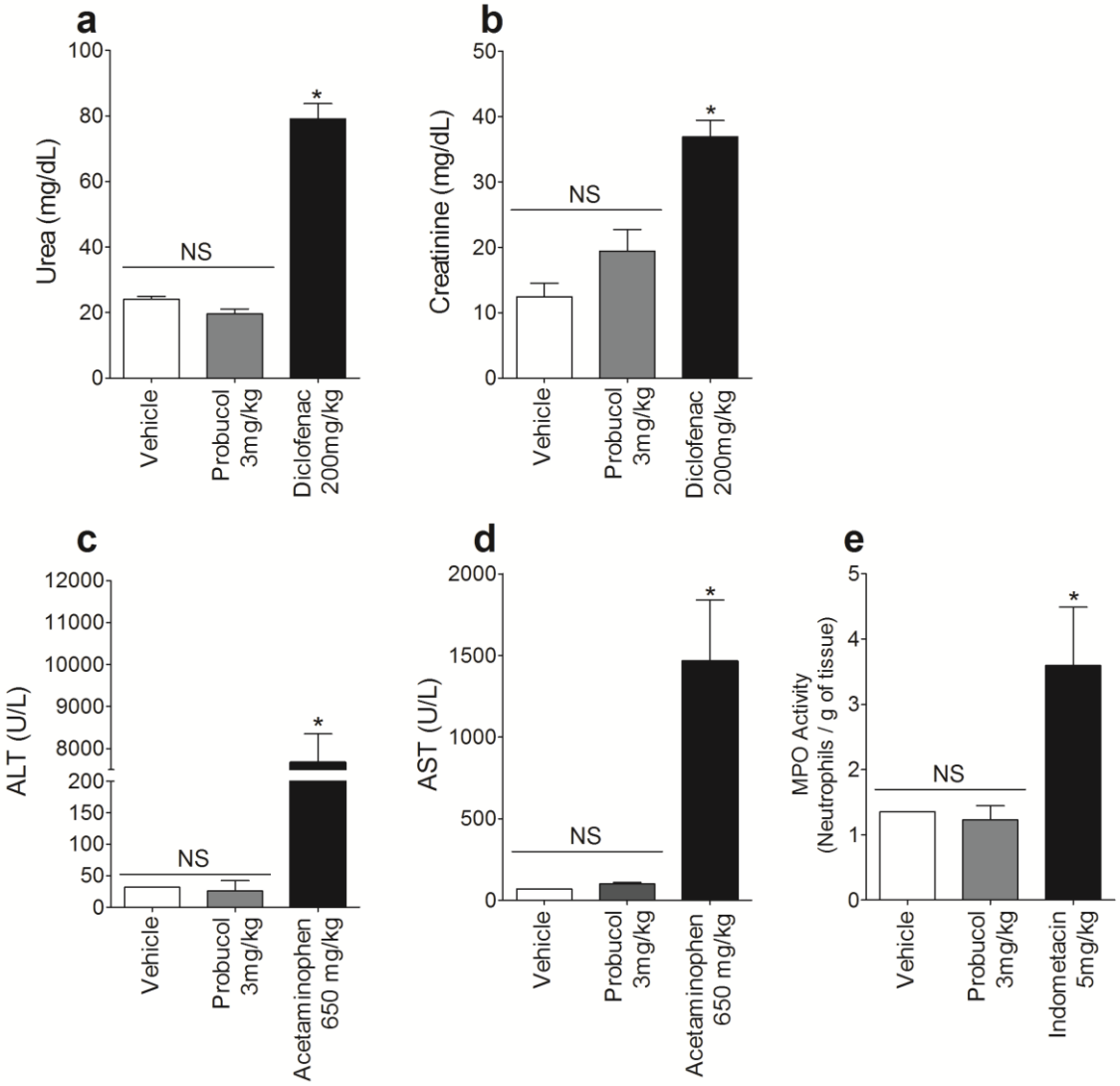
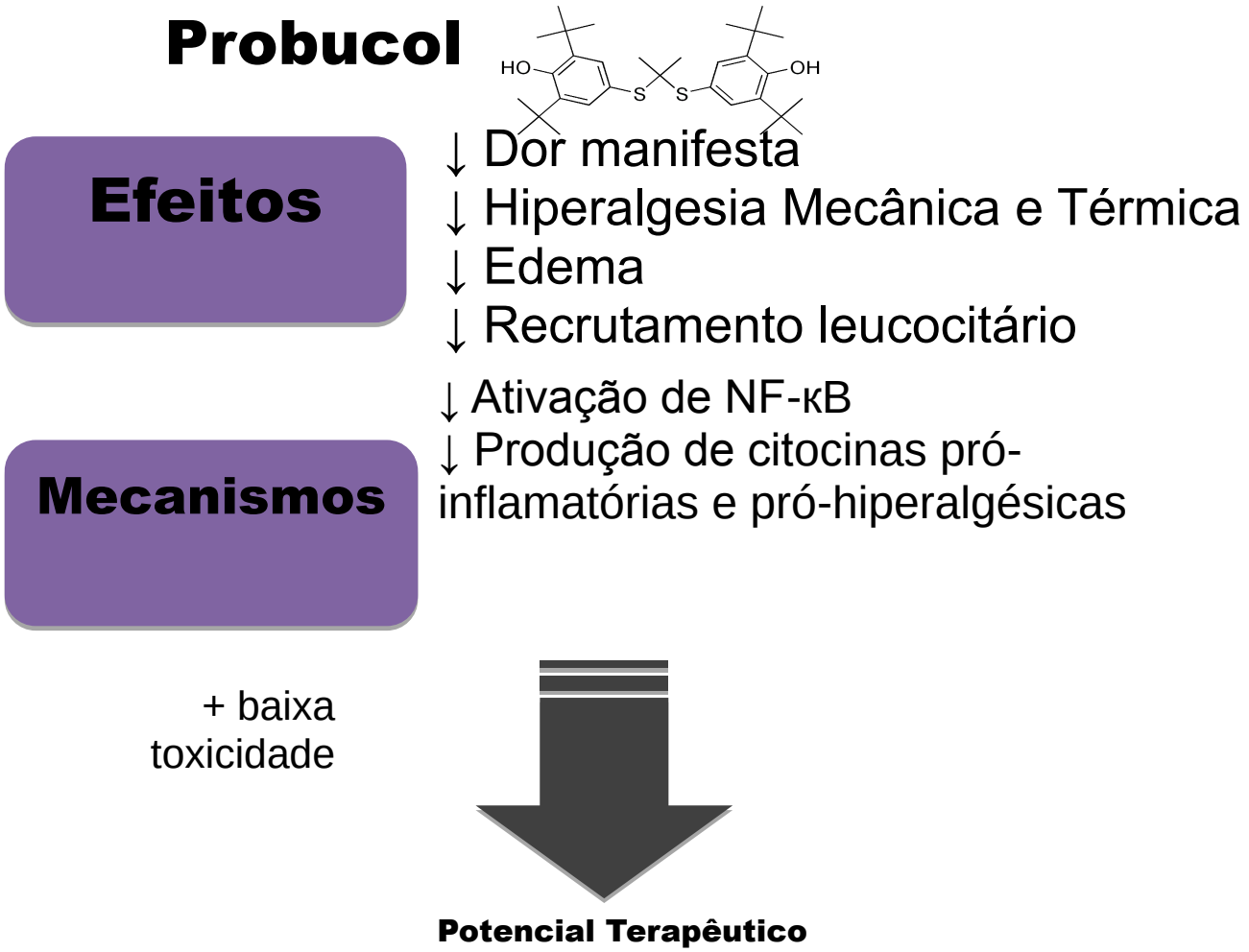


Fig. 7 Treatment with probucol did not affect renal or liver function or caused gastric lesions. Mice received probucol at 3 mg/kg (p.o.) daily for 7 days. Diclofenac (200 mg/kg, single dose, 24h) was used as a positive control for nephrotoxicity. Acetaminophen (650 mg/kg, single dose, 10 h) was used as positive control for hepatotoxicity. Indomethacin (5 mg/kg, daily for 7 days) was used as a positive control for gastric lesion. Plasma was obtained to evaluate levels of urea, creatinine, ALT and AST using commercial diagnostic kids. Stomach was removed to evaluate MPO activity. Results are expressed as mean ± SEM (n = 6 per group per experiment, representative of two separate experiments). One-way ANOVA followed by Tukey's post-hoc. *$p<0.05$ vs. vehicle group.

6 CONCLUSÃO

Probucol foi introduzido no mercado para o tratamento de hipercolesterolemias. No entanto, estudos clínicos demonstraram seu efeito negativo sobre os níveis de HDL, o que comprometeu ou impediu sua comercialização em muitos países ocidentais. Posteriormente, seus efeitos anti-inflamatórios e antioxidantes foram demonstrados *in vitro* e em modelos experimentais de diversas doenças inflamatórias crônicas como aterosclerose, lesão isquêmica, diabetes mellitus e doenças neurodegenerativas. Dessa forma, a fim de investigar os mecanismos anti-inflamatórios e analgésicos de probucol utilizamos modelos clássicos de inflamação aguda por carragenina e lipopolissacarídeo e demonstramos o efeito inibitório de probucol sobre a via clássica do NF-κB e sobre a cascata de citocinas pró-hiperalgésicas, contribuindo, assim, para a descrição de seus alvos moleculares. O efeito de probucol em modelo de inflamação sub-aguda induzida por ajduvante completo de Freund também foi benéfico, com inibição de citocinas pró-inflamatórias e pró-hiperalgésicas. Esses resultados, somados à baixa toxicidade de probucol, contribuem para o esclarecimento de seus mecanismos de ação e ratificam seu potencial terapêutico como anti-inflamatório e analgésico (Fig. 2).

Figura 2 – Fluxograma representativo dos efeitos e mecanismos anti-inflamatórios e analgésicos de probucol



Fonte: o próprio autor.