FELIPE ALMEIDA DE PINHO RIBEIRO

NARINGENINA REDUZ INFLAMAÇÃO E DOR:

INIBIÇÃO DO FATOR NUCLEAR KAPPA B E ATIVAÇÃO DA VIA ANALGÉSICA DO ÓXIDO NÍTRICO

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Trabalho apresentado ao Programa de Pós-Graduação em Patologia Experimental da Universidade Estadual de Londrina como requisito para obtenção do título de doutor em Patologia Experimental.

Orientador: Prof. Dr. Waldiceu Aparecido Verri Junior.

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Londrina, 20 de Junho de 2016.

PINHO-RIBEIRO, Felipe Almeida. **Naringenina reduz inflamação e dor:** efeito sobre o fator nuclear kappa B e via analgésica do óxido nítrico. 2016. 72 f. Tese (Doutorado em Patologia Experimental) – Universidade Estadual de Londrina, Londrina. 2016.

RESUMO

A dor inflamatória gera desconforto e reduz drasticamente a qualidade de vida de pacientes de doenças onde há persistência do processo inflamatório. A baixa eficácia e os efeitos adversos dos tratamentos atuais justificam a necessidade de caracterização de novos compostos com potencial terapêutico e baixa toxicidade. O presente estudo investigou os efeitos da naringenina, um flavonóide presente em grandes quantidades nas frutas cítricas, na redução da dor e da inflamação. Para isso, camundongos Swiss foram tratados com naringenina (16,7 – 150 mg/kg, via oral), antes ou após a aplicação dos estímulos inflamatórios. Foram utilizados estímulos clássicos no estudo da dor inflamatória: ácido acético, parabenzoquinona, formalina, capsaicina, adjuvante completo de Freund, carragenina, prostaglandina E_2, e LPS. Para avaliação da intensidade da dor, foram avaliados os comportamentos de contrações abdominais, sacudidas e lambidas de pata, ou retiradas de pata após estímulo mecânico (analgesímetro digital) ou térmico (placa quente), dependendo do estímulo utilizado e da região estimulada. O efeito da naringenina na produção de citocinas inflamatórias, no estresse oxidativo, e na ativação e recrutamento de leucócitos também foi avaliado após estímulo com carragenina ou LPS. A ativação da via analgésica do óxido nítrico e a inibição da via inflamatória do fator nuclear kappa B (NF-κB) foram avaliadas após estímulo com carragenina e LPS, respectivamente. O tratamento com naringenina foi amplamente eficaz em reduzir a dor inflamatória induzida por todos os estímulos utilizados neste trabalho. Seu efeito foi acompanhado de reduções na produção de citocinas (IL-1β, IL-6, IL-33, IL-12 e TNFα) e na atividade do NF-κB. Houve também redução na ativação e recrutamento de neutrófilos e macrófagos e no estresse oxidativo. Por fim, a inibição da via analgésica do óxido nítrico reverteu os efeitos da naringenina na redução da hiperalgesia. A naringenina não alterou a atividade motora nem a resposta a estímulos dolorosos na ausência de inflamação. O tratamento diário prolongado (7 dias) com naringenina não causou danos gástricos ou hepáticos. Esses resultados sugerem que a naringenina é amplamente eficaz na redução da dor inflamatória através da ativação da via analgésica do óxido nítrico e da inibição da via inflamatória do NF-κB.

Palavras-chave: Naringenina. Dor. Inflamação. Óxido nítrico. NF-κB.

PINHO-RIBEIRO, Felipe Almeida. **Naringenin reduces inflammation and pain:** effect on nuclear factor kappa B and nitric oxide analgesic pathway. 2016. 72 p. Thesis (Doctoral degree in Experimental Pathology) – Universidade Estadual de Londrina, Londrina. 2016.

ABSTRACT

Inflammatory pain generates discomfort and reduces dramatically the quality of life of patients of diseases where there is persistence of inflammatory process. The low efficacy and adverse effects of current treatments justify the need for characterizing new compounds with therapeutic potential and low toxicity. The present study investigated the effects of naringenin, a flavonoid present in large quantities in citrus fruits, in reducing pain and inflammation. Swiss mice were treated with naringenin (16.7 - 150 mg/kg, orally) prior to or after application of the inflammatory stimuli. Classical stimuli in the study of inflammatory pain were used: acetic acid, parabenzoquinone, formalin, capsaicin, Freund's complete adjuvant, carrageenan, prostaglandin E_2, and lipopolysaccharide (LPS). For evaluation of pain intensity, we evaluated the behaviors of abdominal contractions, paw flinching and licking, or paw withdrawal after mechanical (digital analgesymeter) or thermal (hot plate) stimulus, depending on the compound used and on the region stimulated. The effect of naringenin on the production of inflammatory cytokines, oxidative stress, and activation and recruitment of leukocytes was also evaluated after LPS or carrageenan stimuli. Activation of the nitric oxide analgesic pathway and inhibition of nuclear factor kappa B (NF-κB) inflammatory pathway were evaluated after stimulus with carrageenan or LPS, respectively. Treatment with naringenin was broadly effective in reducing the inflammatory pain induced by all stimuli used in this work. Its effect was accompanied by reductions in cytokine production (IL-1β, IL-6, IL-33, IL-12 and TNF) and NF-κB activity. There was also reduction in activation and recruitment of neutrophils and macrophages and in oxidative stress. Finally, inhibition of nitric oxide analgesic pathway reversed the effects of naringenin in reducing hyperalgesia. Naringenin did not affect motor activity or the response to painful stimuli in the absence of inflammation. Prolonged daily treatment (7 days) with naringenin did not cause gastric or liver damage. These results suggest that the naringenin is broadly effective in reducing inflammatory pain through activation of nitric oxide analgesic pathway and inhibition of NF-κB inflammatory pathway.

Keywords: Naringenin. Pain. Inflammation. Nitric oxide. NF-κB.

LISTA DE ILUSTRAÇÕES

SUMÁRIO

1 INTRODUÇÃO

A integridade do organismo depende da habilidade em detectar rapidamente situações de risco e gerar comportamentos que levem a redução da exposição a estas situações. Esta habilidade é garantida pelo sistema nervoso que, nos seres humanos, integra neurônios periféricos especializados na detecção de estímulos intensos (neurônios nociceptores) às áreas do sistema nervoso central para gerar a experiência desagradável de dor. Mesmo assim, danos ao organismo são frequentes e de modo geral não representam risco à sobrevivência do indivíduo. Tendo em vista que lesões teciduais permitem que patógenos (p.ex. bactérias e fungos) entrem em contato direto com o sistema circulatório, a integridade do organismo depende também da habilidade em responder a danos teciduais de modo a evitar que agentes patogênicos colonizem os tecidos expostos. O conjunto de alterações vasculares e celulares que seguem a lesão tecidual caracteriza a resposta inflamatória aguda que, entre outras funções, recruta células do sistema imunológico inato especializadas no combate a patógenos, como neutrófilos e macrófagos. A resposta inflamatória é, contudo, fonte de mediadores que alteram a responsividade dos neurônios nociceptores e aumentam a sensibilidade à dor, chamada dor inflamatória. Em doenças como artrite reumatoide e psoríase, a persistência da resposta inflamatória gera sofrimento constante aos indivíduos e transforma tarefas básicas do cotidiano em situações aversivas.

Apesar de a atividade terapêutica de diversas plantas ser conhecida há muito tempo, pouco se sabe a respeito dos compostos responsáveis por essa atividade. Produtos naturais derivados de plantas, como morfina e salicina, foram essenciais para a construção do conhecimento científico da neurobiologia da dor, e seu uso permitiu o tratamento de doenças e o controle de seus sintomas, incluindo a dor inflamatória. Por este motivo e pela limitada eficácia dos tratamentos disponíveis atualmente, a caracterização biológica/farmacológica desses compostos é fundamentalmente um trabalho necessário para melhorar a qualidade de vida de indivíduos doentes.

1.1 INFLAMAÇÃO

A inflamação consiste em um conjunto de alterações vasculares e celulares desencadeadas após distúrbios da homeostase tecidual e/ou presença de moléculas que indicam dano tecidual ou infecção. Essas alterações são responsáveis pelos quatro sinais

cardinais da inflamação descritos pelo escritor e médico romano Aulus Cornelius Celsus (Século I a.C.): calor e rubor, causados pelo aumento de fluxo sanguíneo local; edema, decorrente do aumento de permeabilidade vascular e acúmulo de líquido nos tecidos; e dor, mais especificamente a dor inflamatória, resultado da atividade de mediadores inflamatórios nos neurônios nociceptores. Com a lesão tecidual, moléculas geralmente encontradas em grandes quantidades no interior das células são liberadas após a ruptura da membrana plasmática. Por serem liberadas após dano celular, essas moléculas são conhecidas como padrões moleculares associados a danos, ou DAMPs (do inglês damage-associated molecular patterns), e incluem a adenosina trifosfato (ATP) e os peptídeos formilados mitocondriais (fMLP). Juntamente com moléculas de origem microbiana como o lipopolissacarídeo (LPS), conhecidas como padrões moleculares associados a patógenos ou PAMPs (do inglês patogen-associated molecular patterns), os DAMPs são inicialmente reconhecidos por células sentinelas que residem no tecido afetado, como macrófagos, células dendriticas e mastócitos (KUMAGAI; AKIRA, 2010; TAKEUCHI; AKIRA, 2010; ZHANG et al., 2010; VOLMERING et al., 2016).

Macrófagos são ativados após o reconhecimento de PAMPs e DAMPs e passam então a produzir moléculas pró-inflamatórias, como citocinas e mediadores lipídicos (Figura 1). Há também a ativação de sistemas geradores de espécies reativas como as enzimas NADPH oxidase 2 (NOX2) e óxido nítrico sintase (NOS) que produzem ânion superóxido e óxido nítrico, respectivamente. Quando combinados, ânion superóxido e óxido nítrico dão origem ao peroxinitrito cuja reatividade com biomoléculas pode ser extremamente prejudicial às funções celulares. A produção desses mediadores é induzida pelo fator de transcrição nuclear kappa B (NF-κB), um ponto de convergência das vias de sinalização pró-inflamatórias clássicas desencadeadas por PAMPs e DAMPs (MCDONALD; BALD; CASSATELLA, 1997; MORGAN; LIU, 2011; OECKINGHAUS; HAYDEN; GHOSH, 2011). Entre os mediadores cuja expressão é induzida pela via do NF-κB, as citocinas interleucina 1 beta (IL-1β), IL-6 e fator de necrose tumoral alfa (TNFα), e o mediador lipídico prostaglandina E_2 (PGE_2) produzido pela enzima ciclo-oxigenase 2 (COX2) não só atuam recrutando e ativando leucócitos (p.ex. neutrófilos e monócitos) mas também ativam diretamente receptores presentes em neurônios nociceptores (TAKEUCHI; AKIRA, 2010; SADIK; KIM; LUSTER, 2011; VERRI JR. et al., 2006). Este efeito direto dos mediadores inflamatórios nos neurônios nociceptores causa modificações de curta e longa duração que facilitam a despolarização e que desencadeiam, consequentemente, a dor inflamatória.

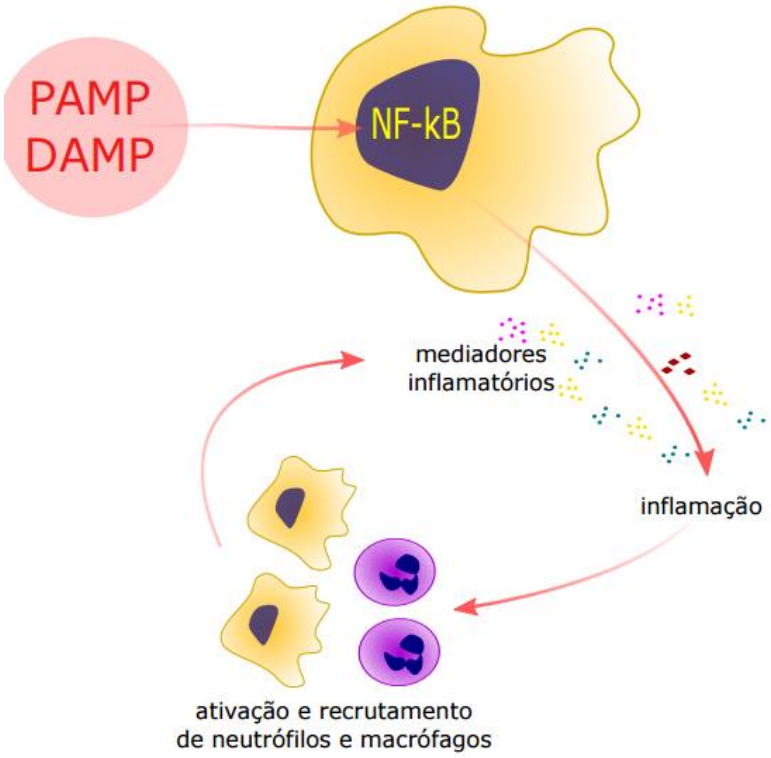

Figura 1. Ativação da via do NF-κB e da resposta inflamatória

1.2 Dor Inflamatória

A dor inflamatória, diferente da dor aguda que gera reflexos de retirada, não desaparece com a retirada do estímulo externo, mas tende a reduzir com a resolução do processo inflamatório. Esta característica reflete o papel dos mediadores inflamatórios no processo de sensibilização neuronal. Como citado anteriormente, os neurônios nociceptores de primeira ordem, cujas terminações nervosas encontram-se em maior quantidade nos tecidos e órgãos que fazem contato direto com o meio ambiente (p.ex. pele e córnea), possuem receptores para alguns mediadores produzidos pelo sistema imunológico durante a resposta inflamatória (Figura 2). As citocinas IL-1β e IL-6 ativam respectivamente os receptores IL-1R e gp130 presentes nos neurônios nociceptores e aumentam a expressão do receptor de potencial transitório do tipo vanilóide 1 (TRPV1) (EBBINGHAUS et al., 2012; MALSCH et al., 2014; FANG et al., 2015). TRPV1 é um receptor que age como canal iônico que permite o influxo de cálcio quando ativado por temperaturas nocivas (acima de 43 ^{o}C) ou por agonistas (p.ex. capsaicina e prótons). Com o aumento na expressão de TRPV1 induzido por IL-1β e IL-6, o número de receptores ativados simultaneamente aumenta e o influxo de

cálcio é maior. Com isso, a despolarização da membrana neuronal é potencializada e o limiar de ativação e abertura dos canais de sódio é atingido mais facilmente, um processo conhecido como sensibilização periférica que tem como resultado o aumento na percepção da dor. Já a citocina TNFα atua nos neurônios nociceptores através do receptor TNFRI para induzir a fosforilação de canais de sódio NaV1.9 responsáveis pela propagação do impulso nervoso. A fosforilação desses canais reduz o limiar de ativação e atrasa a etapa de inativação, permitindo assim que os canais sejam ativados mais facilmente e que sua atividade permaneça por mais tempo (GUDES et al., 2015).

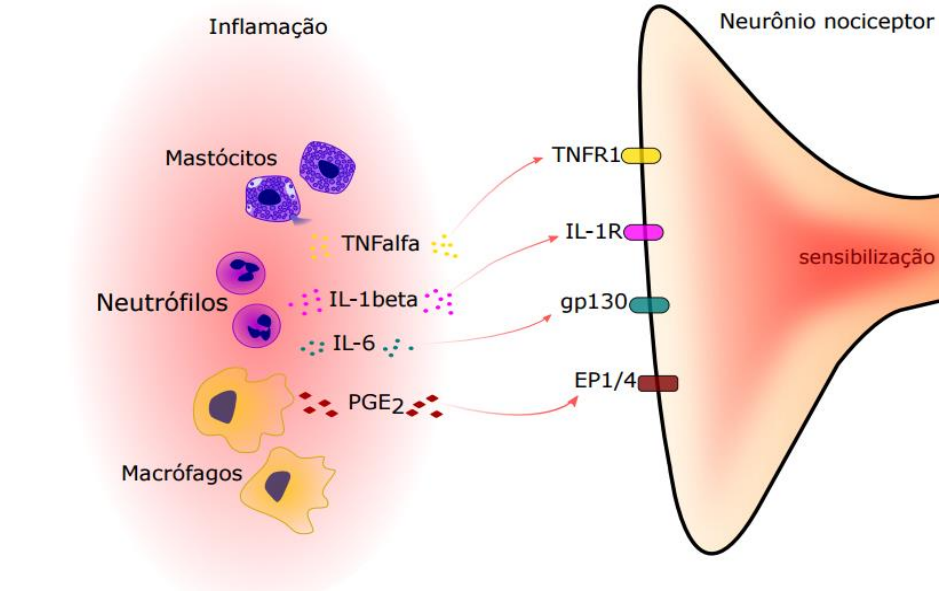

Figura 2. Sensibilização periférica

A participação da PGE_2 na dor inflamatória é facilmente deduzida se considerarmos que inibidores da COX representam a classe de medicamentos mais utilizados para controle da dor inflamatória, os anti-inflamatórios não-esteroidais. Mesmo assim, os mecanismos pelos quais a PGE_2 atua nos neurônios nociceptores para aumentar a sensibilidade à dor ainda não estão totalmente compreendidos. Sabe-se, porém, que a PGE_2 induz a sensibilização periférica através dos receptores EP1 e EP4. A ativação de EP1 tem como resultado a liberação de cálcio dos estoques internos, enquanto a ativação de EP4 gera um feedback positivo que aumenta a quantidade de receptores EP4 (ST-JAQUES; MA, 2016). Além desta via de auto-potencialização da PGE_2, há também um efeito induzido pelas citocinas TNFα e IL-1β conhecido como priming hiperalgésico no qual a responsividade neuronal à PGE_2 é

aumentada cerca de 3 dias após a atuação das citocinas. Esse efeito depende da ativação de fatores de transcrição neuronais (p.ex. CREB) e da expressão e transporte de RNAm e proteínas até as terminações nervosas periféricas e espinais (FERRARI et al., 2015). O intervalo de tempo necessário para que este efeito ocorra é resultado da distância entre os terminais nervosos e o núcleo dos neurônios nociceptores que se encontram próximos à medula espinal, nos gânglios da raiz dorsal (DRG). As alterações estruturais que caracterizam o priming hiperalgésico sustentam a sensibilização neuronal por um período longo e favorecem a cronicidade da dor inflamatória.

A participação de outros mediadores além da PGE_2 especialmente em doenças caracterizadas pela inflamação persistente (p.ex. artrite reumatoide) contribui para a eficácia limitada na redução da dor com anti-inflamatórios não-esteroidais que atuam inibindo a atividade da COX e a produção de PGE_2. Além disso, a utilização contínua de compostos inibidores da COX leva ao desenvolvimento de lesões gástricas que reduzem a qualidade de vida dos indivíduos e representam a principal causa de intoxicação por medicação (PAYNE, 2000; PARADA et al., 2003; FATTORI; AMARAL; VERRI JR, 2016). Analgésicos opióides, por outro lado, geram graves efeitos colaterais e rápida tolerância, além de não serem eficazes na redução do processo inflamatório (PAYNE, 2000). Compostos com atividades anti-inflamatória e analgésica por mecanismos distintos dos atualmente utilizados são possíveis candidatos a substituir os tratamentos convencionais. Muitos compostos naturais do grupo dos polifenóis não só apresentam tais atividades como carecem de efeitos colaterais expressivos e, dessa forma, despertam o interesse da pesquisa biomédica (PINHO-RIBEIRO et al., 2015; FATTORI et al., 2015).

1.3 NARINGENINA

Naringenina é uma flavanona, um dos primeiros compostos na via de síntese dos flavonoides. Sua produção ocorre após a ciclização da chalcona pela enzima chalcona isomerase (CHI), e serve de substrato para síntese de quase todos os demais flavonoides (Figura 3) (MA et al., 2009; KANG et al., 2014). A naringenina ocorre principalmente na forma de heterosídeo (naringina) no pericarpo de frutas cítricas, como grapefruit, tangerina e laranja, mas está presente também no tomate, e sua concentração pode chegar a 50 mg/100 g de fruta no caso da grapefruit (RANKA et al., 2008). Após a ingestão de frutas cítricas esses valores podem aumentar já que o heterosídeo naringina, responsável pelo sabor amargo da

grapefruit, é metabolizado pela microbiota intestinal gerando mais naringenina. A naringenina é detectada no sangue 20 min após administração oral em humanos, atinge seu pico plasmático 3h e 30 min após, e sua meia vida é de 2h, sendo o pico da excreção 4h e 30 min. A presença de naringenina 20 min após ingestão por humanos sugere absorção nas porções iniciais do intestino ou mesmo no estômago (pH ácido). Em ratos, foram observados dois picos plasmáticos, 15 min e 3h após ingestão. Esse segundo pico é resultado de reabsorção do conteúdo excretado pela bile (MA et al., 2009; KANG et al., 2014).

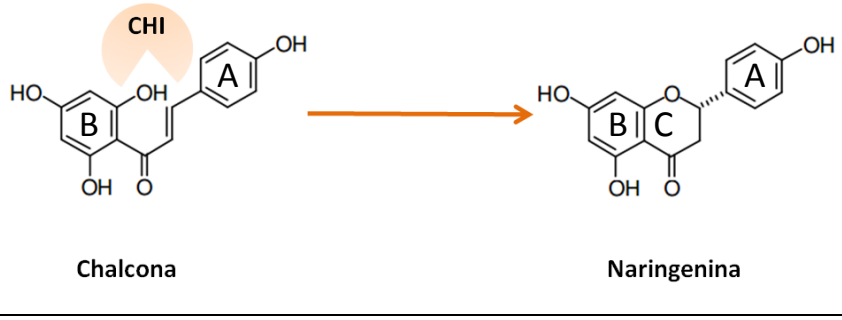

Figura 3. Conversão de chalcona em naringenina pela chalcona isomerase (CHI)

O papel da naringenina nos vegetais, assim como o de outros metabólitos secundários da via dos flavonóides, ainda não está bem definido, porém sabe-se que a planta pode utilizar estes compostos para sua coloração, proteção contra radiação ultravioleta (duplas conjugadas) e controle de patógenos (HARBORNE; WILLIAMS, 2000). Contudo, o papel de flavonóides em uma espécie de planta não necessariamente reflete seu papel nas demais espécies, assim como de outros metabólitos em diferentes seres vivos. O consumo da naringenina, por outro lado, apresenta diversos efeitos benéficos que suportam o seu potencial terapêutico. Assim como outros compostos polifenólicos, a naringenina tem atividade antioxidante e anti-inflamatória, além de auxiliar no tratamento da hipertensão, diabetes, esteatose hepática, entre outras doenças (AHMED et al., 2014; XING et al., 2016; ASSINI et al., 2015). Nosso grupo observou recentemente que a naringenina administrada sistemicamente ou topicamente previne a inflamação e o estresse oxidativo cutâneo causado por radiação ultravioleta B (MARTINEZ et al., 2015; 2016), sugerindo que a naringenina, e não seus metabólitos, é o princípio ativo responsável pelos efeitos benéficos observados. Nesses estudos, também observamos que a naringenina apresenta baixa atividade sequestradora de radicais livres (IC50 = 672 µM para radicais hidroxil), e que seus efeitos antioxidantes e anti-inflamatório estão relacionados à potencialização da via do Nrf2. Considerando que a atividade da via do Nrf2 modula negativamente a ativação do NF-κB, e que sua atividade inibitória nesta última

via já foi demonstrada em células do sistema imunológico como macrófagos (PARK et al., 2012), é possível que estes efeitos resultem em inibição da dor inflamatória.

Efeitos neuronais da naringenina também têm sido descritos recentemente. Além de neuroprotetora, a naringenina modula a atividade de canais de potássio, aumentando o fluxo de potássio e diminuindo a atividade de neurônos motores (HSU et al., 2014). Em neurônios nociceptores, a ativação dos canais de potássio sensíveis a ATP (K_{ATP}) representa um importante mecanismo analgésico utilizado por compostos como morfina e dipirona, e há relatos de que a naringenina potencializa os efeitos da morfina (LORENZETTI; FERREIRA, 1996; CUNHA et al., 2010). A ativação desta via depende da produção de óxido nítrico (NO) pelos neurônios nociceptores e subsequente ativação de guanilato ciclase (Gc) e proteína kinase G (PKG) que modula a abertura dos K_{ATP} (Figura 4). De fato, os efeitos analgésicos da naringenina já foram relatados por outros grupos em modelos de neuropatia e correlacionados à redução da neuroinflamação e da ativação da glia, sugerindo que a naringenina possa apresentar efeito modulatório direto da atividade neuronal (HU; ZHAO, 2014; AL-REJAIE et al., 2015). Dessa forma, investigamos neste trabalho o potencial terapêutico da naringenina no tratamento da dor inflamatória e sua relação com a modulação da via pró-inflamatória do NF-κB e da via analgésica do NO.

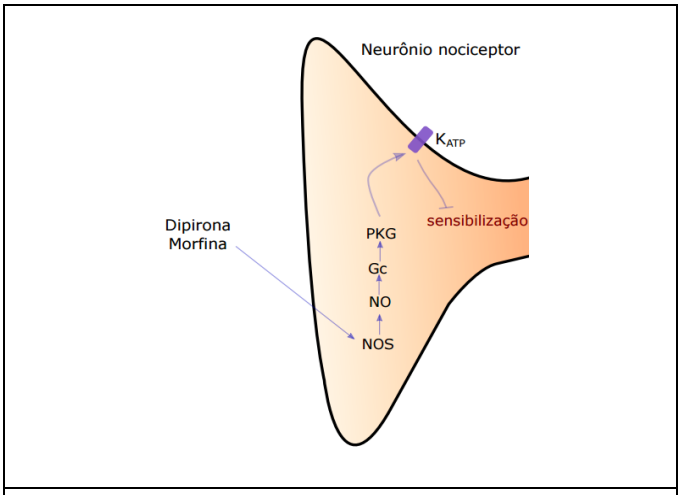

Figura 4. Via analgésica do óxido nítrico

2 OBJETIVOS

2.1 Objetivo Geral

Avaliar o potencial terapêutico da naringenina na redução da dor e inflamação.

2.2 Objetivos Específicos

- Avaliar o efeito do tratamento com naringenina na redução da dor aguda e da hiperalgesia induzidas por agentes inflamatórios;

- Avaliar o efeito da naringenina na ativação e recrutamento de neutrófilos e macrófagos após estímulo inflamatório;

- Avaliar o efeito da naringenina na produção de citocinas inflamatórias e atividade do fator de transcrição NF-κB;

- Verificar a dependência da via analgésica do NO nos efeitos da naringenina

3 ARTIGOS

Este trabalho foi desenvolvido no Laboratório de Dor e Inflamação da Universidade Estadual de Londrina. Os resultados obtidos encontram-se publicados em dois artigos científicos:

1) *Naringenin reduces inflammatory pain in mice*. Pinho-Ribeiro FA, Zarpelon AC, Fattori V, Manchope MF, Mizokami SS, Casagrande R, Verri WA Jr. Neuropharmacology. 2016 Jun; 105:508-19.

2) *The citrus flavonone naringenin reduces lipopolysaccharide-induced inflammatory pain and leukocyte recruitment by inhibiting NF-κB activation*. Pinho-Ribeiro FA, Zarpelon AC, Mizokami SS, Borghi SM, Bordignon J, Silva RL, Cunha TM, Alves-Filho JC, Cunha FQ, Casagrande R, Verri WA Jr. J Nutr Biochem. 2016 Jul;33:8-14.

As formatações dos artigos seguem as normas das revistas *Neuropharmacology* e *Journal of Nutritional Biochemistry*.

Naringenin reduces inflammatory pain in mice

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Highlights

- Naringenin inhibited pain behavior induced by varied inflammatory stimuli.
- Naringenin mechanisms involved inhibition of oxidative stress.
- Naringenin also inhibits IL-33, TNF-α and IL-1β production.
- Naringenin inhibits NFκB activation.
- Naringenin activates the NO-cyclic GMP-PKG-ATP sensitive K^+ channel pathway.

Abstract

Naringenin is a flavonoid widely consumed by humans that present anti-inflammatory activity and low toxicity. Recently, the analgesic effect of naringenin has been demonstrated in neuropathic pain models. Herein, we tested the analgesic effects of naringenin in several models of inflammatory pain. Mice received treatment with naringenin (16.7–150 mg/kg, per oral), or with the controls anti-inflammatory drugs indomethacin (5 mg/kg, intraperitoneal) or dipyrone (80 mg/kg, intraperitoneal) prior the inflammatory stimuli injection. For acute pain, we used acetic acid- and PBQ-induced visceral pain (abdominal writhings), and formalin-, capsaicin-, and CFA-induced paw flinching and licking. By using an electronic version of von Frey filaments, we also investigated the effects of naringenin in pain intensity to a mechanical stimulus (mechanical hyperalgesia) after carrageenan, capsaicin, CFA, or PGE_2 intraplantar injection. Naringenin (50 mg/kg) reduced acute pain behaviors induced by all tested stimuli, including both phases of formalin test, suggesting a direct nociceptor modulatory effect of this compound besides its anti-inflammatory activity. Accordingly, naringenin also inhibited the increased sensitivity to mechanical stimulus induced by carrageenan, capsaicin, and PGE_2. Daily treatment with naringenin during 7 days also reduced CFA-induced mechanical hyperalgesia without gastric or hepatic toxicity. The mechanisms of naringenin involve the inhibition of carrageenan-induced oxidative stress, hyperalgesic cytokines (IL-33, TNF-α, and IL-1β) production and NF-κB activation in the paw skin. Naringenin also activated the analgesic NO-cyclic GMP-PKG-ATP sensitive K^+ channel signaling pathway to inhibit carrageenan-induced mechanical hyperalgesia and neutrophil recruitment. These results suggest that naringenin inhibits both inflammatory pain and neurogenic inflammation.

Keywords
Overt-pain; Carrageenan; Hyperalgesia; Cytokines; NF-κB

1. Introduction

Pain is a response to stimuli with actual or potential tissue damage and represents an essential adaptive response to protect the integrity of the organism. However, pain can also be generated by maladaptive responses of the organism, affecting daily activities and quality of life. Persistence and exacerbation of pain can occur due to sensitization of nociceptors,

somatosensory neurons that respond to noxious stimuli and represent an important step for pain generation. IL-33 induces the production of tumor necrosis factor alpha (TNFα), IL-1β and prostaglandin E_2 (PGE_2), which are inflammatory mediators that act on nociceptive neurons to facilitate their activation and increase pain sensitivity, a state named hyperalgesia (Binshtok et al., 2008, Gudes et al., 2015, St-Jacques and Ma, 2014, Verri et al., 2006 and Zarpelon et al., 2013). This sensitization makes pain experience more unpleasant than usual. Due to the crucial role of inflammatory mediators in hyperalgesia, the use of anti-inflammatory drugs for pain treatment is a common clinical practice that presents some limitations and side effects that increase with the chronicity of its use (Suleyman et al., 2007).

Natural compounds have been essential tools for our understanding of pain mechanisms due to their powerful activity in modulating neuronal activity (e.g. morphine, capsaicin, salicylate, and menthol) (Julius, 2013). Naringenin (4′,5,7-thrihydroxyflavanone) is among the most consumed flavonoids by humans and is easily detected in the human serum after its intake due to its good bioavailability (Palma-Duran et al., 2015). As a flavonoid, naringenin has antioxidant and anti-inflammatory activities, and low toxicity and thus has potential to be used as a therapeutic tool (Cavia-Saiz et al., 2010 and Martinez et al., 2015). Accordingly, the analgesic and neuroprotective effects of naringenin were reported previously by others in models of neuropathic pain and neurodegenerative disorders (Al-Rejaie et al., 2015, Hasanein and Fazeli, 2014, Hu and Zhao, 2014 and Kaulaskar et al., 2012). Naringenin is an activator of transcription factor Nrf2 and agonist of aryl hydrocarbon receptor, two mechanisms that contribute to reducing the production of reactive oxygen species and inflammatory mediators (Guo et al., 2015 and Lou et al., 2014). Importantly, reactive oxygen species and inflammatory mediators sustain their own production by activating the transcription factor NF-κB and contribute synergistically to increase pain sensation by acting on nociceptive neurons (Binshtok et al., 2008, Cuzzocrea et al., 2004, Fattori et al., 2015, Jin and Gereaut, 2006 and Yamacita-Borin et al., 2015).

Despite its well-known anti-inflammatory activity, the benefits of using naringenin to reduce inflammatory pain are still unknown. In this study, we investigated the effects of naringenin treatment in different models of inflammatory pain and addressed its relation with the production of inflammatory hyperalgesic mediators. Our results suggest that naringenin is a promising safe compound in the treatment of inflammatory pain and provide evidence that its mechanisms of action include inhibition of inflammatory mediators by targeting NFκB

activation as well as activation of the NO-cyclic GMP-PKG-ATP sensitive K+ channel pathway.

2. Material and methods

2.1. Animals

Male Swiss mice (20–25 g), from the Universidade Estadual de Londrina, Londrina, Paraná, Brazil, were used in this study. Mice were housed in standard clear plastic cages with free access to food and water, a light/dark cycle of 12:12 h, and kept at 21 °C. All behavioral testing was performed between 9 a.m. and 5 p.m. in a temperature-controlled room (21 °C). Animal care and handling procedures were in accordance with the International Association for Study of Pain (IASP) guidelines and approved by the Ethics Committee of the Universidade Estadual de Londrina (process number 32816.2012.64). Every effort was made to minimize the number of animals used and their suffering.

2.2. Drugs and stimuli

The following materials were obtained from the sources indicated: Saline (NaCl 0.9%) from Fresenius Kabi Brasil Ltda (Aquiraz, CE, Brazil), Naringenin at 95% purity, carrageenan and 1H-(1,2,4)-oxadiazolol-(4,3-a)quinoxalin-1-one (ODQ) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, United States), acetic acid and formaldehyde from Mallinckrodt Baker S.A. (Mexico, Mexico City). Dimethyl sulfoxide (DMSO), dipyrone, Tween 80, complete Freund's adjuvant (CFA), phenyl-p-benzoquinone (PBQ), prostaglandin E_2 (PGE_2), NG-nitro- l-arginine methyl ester (l-NAME) and glibenclamide were obtained from Sigma–Aldrich (St. Louis, MO, USA). 2,3,9,10,11,12-hexahydro-10*R*-methoxy-2,9-dimethyl-1-oxo-9 *S*, 12*R*-epoxy-1diindolo [1,2,3-fg:3′,2′, 1′-kl] pyrrol [3,4-i][1,6]benzodiazocine-10-carboxylic acid, methyl ester (KT5823) from Cayman Chemical Company (Ann Arbor, MI, USA). Indomethacin from Prodome (Campinas, SP, Brazil).

2.3. Writhing response tests

Naringenin (16.7–150 mg/kg, p.o.) was administered 30 min before the nociceptive stimulus at the indicated doses. PBQ and acetic acid-induced visceral pain models were performed as previously described (Verri et al., 2008). PBQ (diluted in 2% DMSO in saline, 1890 μg/kg), acetic acid (0.8% v/v in saline, 10 mL/kg), or vehicle was injected into the peritoneal cavity of mice. Each mouse was placed in a large glass cylinder and the intensity of visceral pain quantified by counting the total number of abdominal writhing occurring between 0 and 20 min after stimulus injection. The writhing response consisted of a contraction of the abdominal muscle together with a stretching of hind limbs. The intensity of the writhing response was expressed as the cumulative number of writhing over 20 min.

2.4. Formalin test

Naringenin (50 mg/kg, p.o.) was administered 30 min before nociceptive stimulus. The number of paw flinching and time spent licking the paw were determined between 0 and 30 min after i.pl. injection of 25 μL of formalin 1.5% as previously described (Borghi et al., 2013). The period was divided in intervals of 5 min, and clearly demonstrated the presence of the first and second phases, which are characteristic of the method. Results were presented as first (0–5 min) and second phase (10–30 min).

2.5. Capsaicin- and CFA-induced overt pain-like behavior

Naringenin (50 mg/kg, p.o.) was administered 30 min before the nociceptive stimulus. Paw flinching and licking were evaluated between 0 and 30 min after i.pl. injection of CFA (10 μL/paw), or between 0 and 5 min after i.pl. injection of capsaicin (1.6 μg/25 μL/paw) (Borghi et al., 2013).

2.6. Carrageenan-, CFA-, capsaicin- and PGE_2-induced mechanical hyperalgesia

Naringenin was administered p.o. 30 min before carrageenan or capsaicin injection at the doses of 16.7–150 and 50 mg/kg, respectively. For CFA test, naringenin treatment (50 mg/kg, p.o.) was given daily, starting one day after CFA injection, and the pain threshold was evaluated 30 min after naringenin administration. Mechanical hyperalgesia was evaluated 1, 3 and 5 h after carrageenan (300 μg/25 μL/paw), capsaicin (1.6 μg/25 μL/paw) or PGE_2 (100 ng/25 μL/paw) injection, and 1–7 days after CFA (10 μL/paw) injection as previously described (Cunha et al., 2004). In a quiet room, mice were placed in acrylic cages (12 × 10 × 17 cm) with wire grid floors, 15–30 min before the start of testing. The test consisted of evoking a hindpaw flexion reflex with a hand-held force transducer (electronic anesthesiometer; Insight, Ribeirao Preto, SP, Brazil) adapted with a 0.5 mm2 polypropylene tip. The cut-off was 18 g. The investigator was trained to apply the tip perpendicularly to the central area of the hindpaw with a gradual increase in pressure. The end point was characterized by the removal of the paw followed by clear flinching movements. After the paw withdrawal, the intensity of the pressure was recorded automatically. The value for the response was an average of three measurements. The animals were tested before and after treatment. The results are expressed by delta (Δ) withdrawal threshold (in grams) calculated by subtracting the mean of the measurements made at 1, 3 and 5 h after stimulus from the zero-time (before injection of stimulus, baseline) mean measurements.

2.7. Determination of total protein concentration

Protein concentration of paw skin samples was determined by the Lowry method (Lowry et al., 1951) adapted to a final volume of 200 μL to be measured using 96 well plates. A standard curve of bovine serum albumin was used. Results were used to normalize the data obtained from other tests using paw skin samples.

2.8. Cytokine measurement

Paw skin tissues were dissected 3 h after carrageenan (300 μg/25 μL/paw) injection and homogenized in 500 μL of ice-cold buffer containing protease inhibitors. Samples were centrifuged and the supernatants used to measure cytokine levels by enzyme-linked immunosorbent assay (ELISA) using eBioscience kit according to manufacturer's instructions. The results are expressed as picograms (pg) of cytokine per 100 mg of total protein.

2.9. Reduced glutathione (GSH) levels assay

Samples from paw skin were collected 3 h after carrageenan (300 μg/25 μL/paw) injection and maintained at −80 °C for at least 48 h and then homogenized with 200 μL of 0.02 M EDTA. Homogenates were mixed with 25 μL of 50% trichloroacetic acid, vortexed three times for 15 min, and centrifuged (15 min × 1500 g × 4 °C), and the resulting supernatant added to 200 μL of 0.2 M Tris buffer, pH 8.2, and 10 μL of 0.01 M DTNB. After 5 min of incubation at room temperature, the absorbance was measured at 412 nm against a blank reagent with no supernatant (Staurengo-Ferrari et al., 2014). A standard curve of GSH was used and the results are reported as nmol of GSH per mg of total protein.

2.10. NF-κB activity

Samples from paw skin were collected 3 h after carrageenan (300 μg/25 μL/paw) injection and homogenized in ice-cold lysis buffer (Cell Signaling). Homogenates were centrifuged (200 g × 10 min × 4 °C) and the supernatants used to assess the levels of phosphorylated and total NF-κB p65 subunit by ELISA (PathScan, Cell Signaling) according to the manufacturer's directions. Absorbance was measured at 450 nm (Multiskan GO Thermo Scientific) and the results are presented as ratio (phospho-p65/total-p65) per mg of total protein.

2.11. Measurement of motor performance

In order to discard possible non-specific muscle relaxant or sedative effects of naringenin, motor performance was evaluated on the rota-rod test (Valerio et al., 2007). The apparatus consisted of a bar with a diameter of 2.5 cm, subdivided into six compartments by disks 25 cm in diameter (Ugo Basile, Model 7600). The bar rotated at a constant speed of 22 rotations per min. The animals were selected 24 h previously by eliminating those mice that did not remain on the bar for two consecutive periods of 120 s. Animals were treated with vehicle (saline) or naringenin (50 mg/kg, p.o.), and testing was performed 1.5, 3.5 and 5.5 h after treatment. It was used a cut-off time of 120s.

2.12. Hepatotoxicity

To investigate the potential toxicity caused by naringenin, mice were treated daily with naringenin, indomethacin, or vehicle for 7 days. After 7 days of treatment, the animals were anesthetized prior to blood collection into heparin-containing tubes. Plasma was obtained by centrifugation at 0.4 g for 10 min. Plasma AST and ALT activities were used as biochemical markers for early acute hepatic damage. These assays were performed using a diagnostic kit from Labtest (Lagoa Santa, MG, Brazil) (Mizokami et al., 2012).

2.13. Myeloperoxidase activity (MPO)

Gastric lesions and neutrophil recruitment were evaluated by the MPO activity kinetic-colorimetric assay (Mizokami et al., 2012 and Staurengo-Ferrari et al., 2014). Briefly, paw skin (5 h after carrageenan injection) and stomach samples (after 7 days of treatment) were collected and homogenized in 50 mM K_2PO_4 buffer (pH 6.0) containing 0.5% HTAB using Ultra-Turrax® (IKA Labortechnik, Staufen, Germany). Homogenates were centrifuged at 16,100 g for 2 min at 4 °C and 7 μL of resulting supernatant was mixed with 200 μL of 50 mM phosphate buffer, pH 6.0, containing 0.167 mg/mL o-dianisidine dihydrochloride and 0.05% hydrogen peroxide. MPO activity was determined at 450 nm (Multiskan GO Microplate Spectrophotometer, ThermoScientific, Vantaa, Finland). MPO activity of samples

were compared with a standard curve of neutrophils and presented as MPO activity (number of neutrophils × 10^4/mg of total protein).

2.14. Statistical analyzes

Results are representative of 6 mice per group per experiment, and two independent experiments making a total of 12 mice per group. Results are mean ± S.E.M. Two-way analysis of variance (ANOVA) followed by Tukey's post hoc was used to analysis in multiple time points. Alternatively, when analyzes were in single time point differences between responses were evaluated by one-way ANOVA followed by Tukey's post hoc. All statistical analyzes were performed using Graph Pad Prism (La Jolla, 5 CA). Statistical differences were considered to be significant at $P < 0.05$.

3. Results

Naringenin inhibits the writhing response induced by acetic acid and phenyl-p-benzoquinone (PBQ). In the first series of experiments, the antinociceptive effect of naringenin was assessed in acetic acid- and PBQ-induced visceral pain (Fig. 1). Mice were treated with naringenin (16.7, 50 and 150 mg/kg, p.o., 30 min) before i.p. stimulus with acetic acid (Fig. 1A) or PBQ (Fig. 1B). Indomethacin (5 mg/kg, i.p.) was used as an anti-inflammatory control drug. All three doses of naringenin reduced the number of abdominal writhings significantly (Fig. 1A) but the effect was more evident in the groups treated with the doses of 50 and 150 mg/kg (Fig. 1A). Since no differences were found between the two higher doses, the dose of 50 mg/kg was used in the experiments with overt pain-like behavior models. In a subsequent experiment, mice were treated p.o with naringenin (50 mg/kg) 30 min before i.p. stimulus with PBQ (1890 μg/kg) (Fig. 1B). The PBQ-induced writhing response was significantly inhibited by naringenin. Indomethacin reduced the writhing response in both models (Fig. 1A and B).

Naringenin inhibits paw flinching and licking induced by formalin, capsaicin, and Complete Freund's Adjuvant (CFA). Mice were treated with naringenin (50 mg/kg, p.o),

indomethacin (5 mg/kg, i.p.) or vehicle before formalin stimulus (1.5%, 25 μL). Naringenin inhibited paw flinching (Fig. 1C) and licking (Fig. 1D) in both phases of the formalin test while indomethacin was able to inhibit these responses in the second phase only (Fig. 1C and D). We next investigated the effect of naringenin in inhibiting the acute pain response induced by capsaicin (TRPV1 agonist). The paw flinching (Fig. 1E) and the time spent licking the paw (Fig. 1F) induced by capsaicin were significantly inhibited by naringenin and indomethacin. In complete Freund's adjuvant (CFA; 10 μL/paw)-induced overt pain-like behavior, pre-treatment with naringenin inhibited both paw flinching (Fig. 1G) and licking behavior (Fig. 1H. Treatment with control drug indomethacin inhibited the paw flinching and licking in the second phase of formalin test, capsaicin and CFA inflammation (Fig. 1).

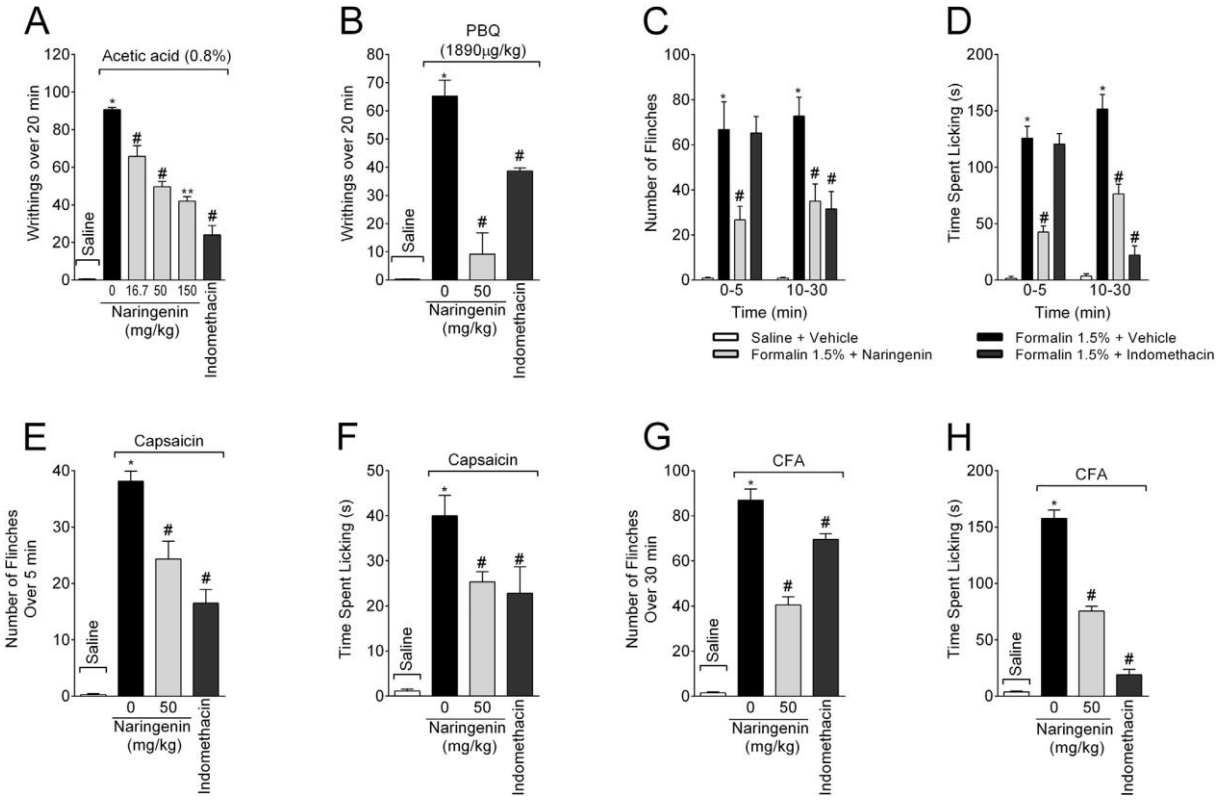

Fig. 1. Naringenin inhibits overt pain-like behavior induced by acetic acid, phenyl-p-benzoquinone (PBQ), formalin, capsaicin, and complete Freund's adjuvant (CFA). Mice were treated with naringenin (16.7, 50, 150 mg/kg, p.o., 30 min), vehicle (saline, p.o., 30 min), or indomethacin (5 mg/kg, i.p., 40 min), before acetic acid (0.8% in saline, i.p.) (A), PBQ (1890 μg/kg in 2% DMSO in saline, i.p.) (B), formalin (25 μL of 1.5% formalin in saline, i.pl.) (C and D), capsaicin (1.6 μg/paw, i.pl.,) (E and F) or CFA (10 μL/paw, i.pl.) (G and H). The cumulative number of abdominal contortions (writhing score) was evaluated over 20 min (A and B). The total number of paw flinches (C, E and G) and the time spent licking the paw (D, F and G) were evaluated during 5 min in the capsaicin test and 30 min in the formalin and CFA tests. Results are means ± SEM of six mice per group per experiment and are representative of two independent experiments. [*$p < 0.05$ compared to the saline group; #$p < 0.05$ compared to the vehicle; **$p < 0.05$ compared to the dose of 16.7 mg/kg (one-way ANOVA followed by Tukey's post hoc)].

Naringenin inhibits carrageenan-, capsaicin-, and CFA-induced mechanical hyperalgesia. The antinociceptive effect of naringenin was tested on mechanical hyperalgesia induced by carrageenan (100 μg/paw), capsaicin (1.6 μg/paw), or CFA- (10 μL/paw). Pre-treatment with naringenin (16.7–150 mg/kg, p.o., 30 min) inhibited carrageenan-induced mechanical hyperalgesia (Fig. 2A) in a dose-dependent manner. Doses of 50 and 150 mg/kg of naringenin inhibited carrageenan-induced mechanical hyperalgesia at 1, 3 and 5 h after the stimulus, while a dose of 16.7 mg/kg inhibited carrageenan-induced mechanical hyperalgesia only 5 h after stimulus (Fig. 2A). Therefore, the dose of 50 mg/kg was selected for subsequent experiments. The post-treatment (1 h) also inhibited the mechanical hyperalgesia (Fig. 2B) at all times tested. Treatment with naringenin inhibited capsaicin-induced mechanical hyperalgesia 1–5 h after stimulus (Fig. 2C). In another experimental setting, mice were treated daily with naringenin (50 mg/kg, p.o., 30 min) starting 24 h after CFA i.pl. injection for seven days for assessment of mechanical hyperalgesia (Fig. 2D). There was significant inhibition of CFA-induced mechanical hyperalgesia at all time points evaluated for treatment with naringenin (1–7 days after stimulus). The control drug, indomethacin (as in Fig. 1) inhibited carrageenan-, capsaicin- and CFA-induced mechanical hyperalgesia (Fig. 2A). Acute and daily treatment with naringenin 50 mg/kg alone did not interfere with the basal response of mice (Fig. 2).

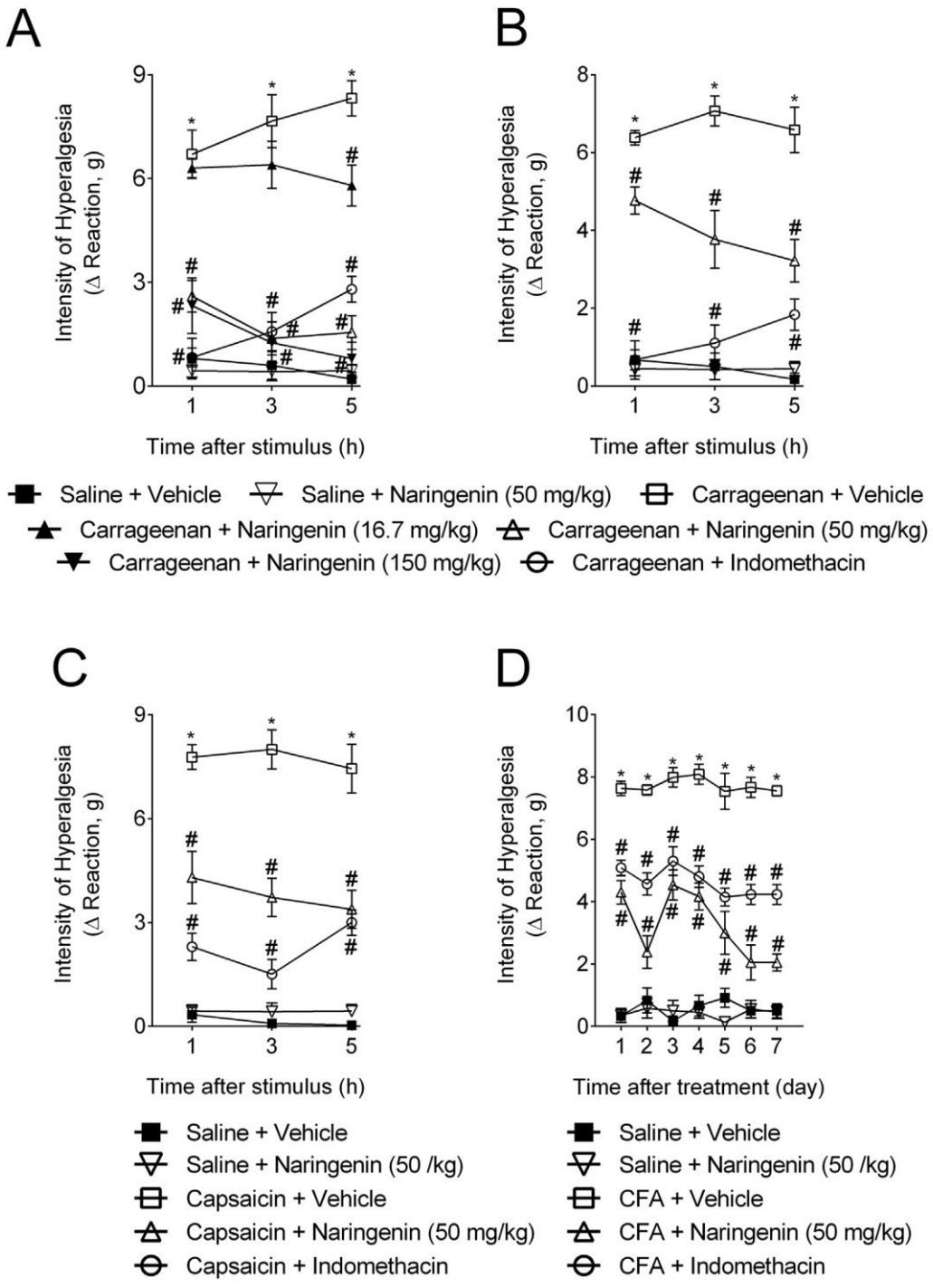

Fig. 2. Treatment with naringenin inhibits carrageenan-, capsaicin-, and CFA-induced mechanical hyperalgesia. Mice were treated with naringenin (16.7–150 mg/kg, p.o., 30 min), vehicle or indomethacin (5 mg/kg, i.p., 40 min) before a carrageenan (300 μg/paw) or saline injection (A). Naringenin was also administered after the carrageenan injection (30 min, B). Mice were also treated with naringenin (50 mg/kg, p.o., 30 min) or vehicle before a capsaicin (1.6 μg/paw, TRPV1 agonist) injection (C) or daily with naringenin (50 mg/kg, i.p.) starting at 24 h after CFA (10 μL/paw) injection (D). The intensity of mechanical hyperalgesia was measured 1–5 h (A–C) or every 24 h (1–8 days) (D) by an electronic pressure-meter test. Results are means ± SEM of six mice per group per experiment, and are representative of two independent experiments. [*$p < 0.05$ compared to the saline group; #$p < 0.05$ compared to the carrageenan group. (Two-way ANOVA followed by Tukey's post hoc)].

Naringenin does not influence motor capacity. Rota-rod test was performed to investigate whether motor impairment, muscle relaxing or hypotensive activity would have an influence in the analgesic of naringenin. Mice (n = 6 per group per experiment, representative of two independent experiments) were treated with vehicle (baseline 116.7 ± 3.3 s) or naringenin (50 mg/kg, p.o., baseline 114.5 ± 5.5 s), and evaluated after 1.5 (117.8 ± 1.6 s, and 116.3 ± 2.3 s, respectively), 3.5 (119.7 ± 0.3 s, and 120.0 ± 0.0 s, respectively), and 5.5 (118.3 ± 1.7 s, and 116.7 ± 0.3 s, respectively) h. The treatment with naringenin did not alter the time that the mice spent walking in the rota-rod disproving motor impairment, muscle relaxing or hypotensive activity.

Naringenin prevents the carrageenan-induced decrease in reduced glutathione (GSH) concentrations. GSH assay in paw tissue were assessed to determinate the antioxidant activity of naringenin (Fig. 3). Mice were treated with naringenin (50 mg/kg, p.o., 30 min, diluted in saline) or indomethacin (5 mg/kg, i.p., 40 min, diluted in tris/HCl buffer) before the i.pl. injection of carrageenan (300 μg/paw), and after an additional 3 h, samples of cutaneous plantar tissue were collected for GSH concentration assay assessment. Carrageenan reduced the endogenous GSH skin levels (Fig. 3), and treatment with naringenin and the control drug prevented these reductions of antioxidant defenses in the inflammatory foci.

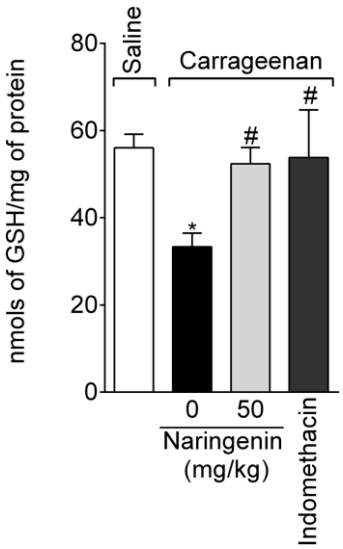

Fig. 3. Naringenin prevents the carrageenan-induced decrease of reduced glutathione (GSH) levels. Mice were treated with naringenin (50 mg/kg, p.o., 30 min), vehicle or indomethacin (5 mg/kg, i.p., 40 min) before a carrageenan injection. Samples of plantar skin tissue were collected 3 h after the stimulus and were processed for GSH assay. Samples of four mice were pooled for processing and the measurements made in three pools per

experiment and are representative of two independent experiments. Total protein was quantified and used to normalize the data from GSH test. [*p < 0.05 compared with the saline group, and #p < 0.05 compared to the vehicle group (One-way ANOVA followed by Tukey's post hoc)].

Naringenin inhibits pro-inflammatory cytokine (IL-33, TNF-α and IL-1β) production induced by carrageenan. Mice were treated with naringenin (50 mg/kg, p.o., 30 min, diluted in saline) or indomethacin (5 mg/kg, i.p., 40 min, diluted in tris/HCl buffer) before the i.pl. injection of carrageenan (300 μg/paw), and after an additional 3 h, samples of cutaneous plantar tissue were collected for cytokine (IL-33, TNF-α, and IL-1β) production determination (Fig. 4). Naringenin and the control drug inhibited carrageenan-induced production of IL-33 (Fig. 4A), TNF-α (Fig. 4B) and IL-1β (Fig. 4C).

Naringenin inhibits carrageenan-induced NF-κB activation. Mice received naringenin (50 mg/kg, p.o., 30 min, diluted in saline) or indomethacin (5 mg/kg, i.p., 40 min, diluted in tris/HCl buffer) before i.pl. injection of carrageenan (300 μg/paw) and samples of the cutaneous plantar tissue were collected 3 h after stimulus injection. Carrageenan-induced activation of NF-κB was observed as an increase of the phosphorylated p65/total p65 ratio while treatment with naringenin as well as the control drug inhibited carrageenan-induced activation of NF-κB (Fig. 4D).

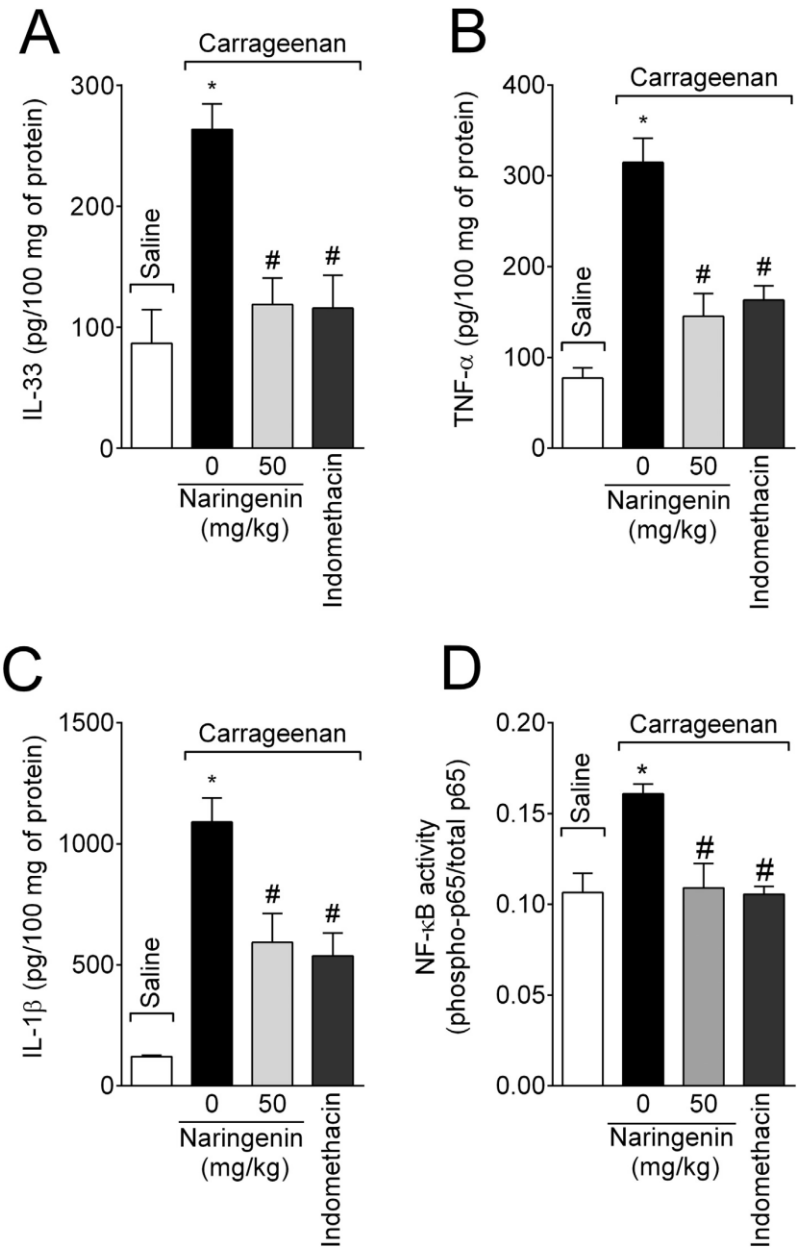

Fig. 4. Naringenin reduces IL-33, TNF-α, IL-1β production and NF-κB activation in carrageenan-induced paw inflammation. Mice were treated with naringenin (50 mg/kg, p.o., 30 min), vehicle or indomethacin (5 mg/kg, i.p., 40 min) before the i.pl. injection of carrageenan. Three hours after a carrageenan injection paw skin samples were collected for the determination of IL-33 (A), TNF-α (B), IL-1β (C) levels and NF-κB activation (D). Results are means ± SEM of six mice per group per experiment and are representative of two independent experiments. Total protein was quantified and used to normalize the data of cytokine and NF-κB tests. [*$p < 0.05$ compared with the saline group, and #$p < 0.05$ compared to the vehicle group (One-way ANOVA followed by Tukey's post hoc)].

Naringenin reduced PGE_2-induced mechanical hyperalgesia. Mice received naringenin (50 mg/kg, p.o., 30 min, diluted in saline) or dipyrone (80 mg/kg, i.p., 30 min, diluted in

saline) before i.pl. injection of PGE_2 (100 ng/paw) (Fig. 5). There was a significant inhibition of PGE_2–induced mechanical hyperalgesia at 3 h (peak of PGE_2-induced mechanical hyperalgesia) by treatment with naringenin or with control drug. Dipyrone was used as a control in this experiment because it is known to inhibit PGE_2-induced mechanical hyperalgesia (Cunha et al., 2004).

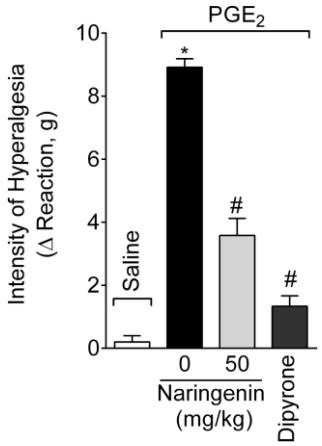

Fig. 5. Naringenin reduces PGE_2-induced mechanical hyperalgesia. Mice were treated with naringenin (50 mg/kg, p.o., 30 min), vehicle or dipyrone (80 mg/kg, i.p., 30 min) before PGE_2 (100 ng/paw) injection. The intensity of mechanical hyperalgesia was measured 3 h after stimulus injection by the electronic pressure-meter test. Results are means ± SEM of six mice per group per experiment and are representative of two independent experiments. [*$p < 0.05$ compared with the saline group, #$p < 0.05$ compared to the vehicle group (One-way ANOVA followed by Tukey's post hoc)].

Naringenin reduced carrageenan-induced mechanical hyperalgesia by activating the NO/GMPc/PKG/ATP sensitive K+channel signaling pathway. Mice were treated with the control drug indomethacin alone (5 mg/kg, i.p.) 40 min before i.pl. injection of carrageenan (300 μg/paw); or with l-NAME (Fig. 6A; l-nitroarginine methyl ester, nonselective NOS inhibitor, 90 mg/kg, i.p., diluted in saline, 60 min), ODQ (Fig. 6B; a soluble guanylate cyclase inhibitor, 0.3 mg/kg, diluted in 2% DMSO in saline, 30 min), KT5823 (Fig. 6C; an inhibitor of PKG, 0.5 μg/animal, diluted in 2% DMSO in saline, 5 min), or glibenclamide (Fig. 6D; an inhibitor of ATP-sensitive potassium channels, 0.3 mg/kg, diluted in 20% Tween 80 in saline, 45 min) before naringenin (50 mg/kg, p.o.) treatment. After an additional 30 min, mice received an i.pl. injection of carrageenan (300 μg/paw), and mechanical hyperalgesia was evaluated after 1, 3, and 5 h. The inhibition of carrageenan-induced hyperalgesia by naringenin was prevented by l-NAME, ODQ, KT5823, and glibenclamide treatments at 1, 3, and 5 h (Fig. 6). Therefore, the antinociceptive effect of naringenin depends, at least in part,

on the induction of NO production and consequent activation of the cyclic GMP− PKG−ATP-sensitive K+ channel signaling pathway, which diminishes the nociceptive neurotransmission, resulting in diminished inflammatory mechanical hyperalgesia. Since activation of this pathway reduces depolarization of a wide group of nociceptive neurons, this activity may benefit thermal hyperalgesia as well (Wang et al., 2008). Furthermore, naringenin inhibited the carrageenan–induced MPO activity, and the treatment with l-NAME, ODQ, KT5823 (Fig. 6E), and glibenclamide (Fig. 6E) also prevented the naringenin inhibition of carrageenan-induced MPO activity (Fig. 6E). Treatment with indomethacin inhibited both hyperalgesia and MPO activity (Fig. 6).

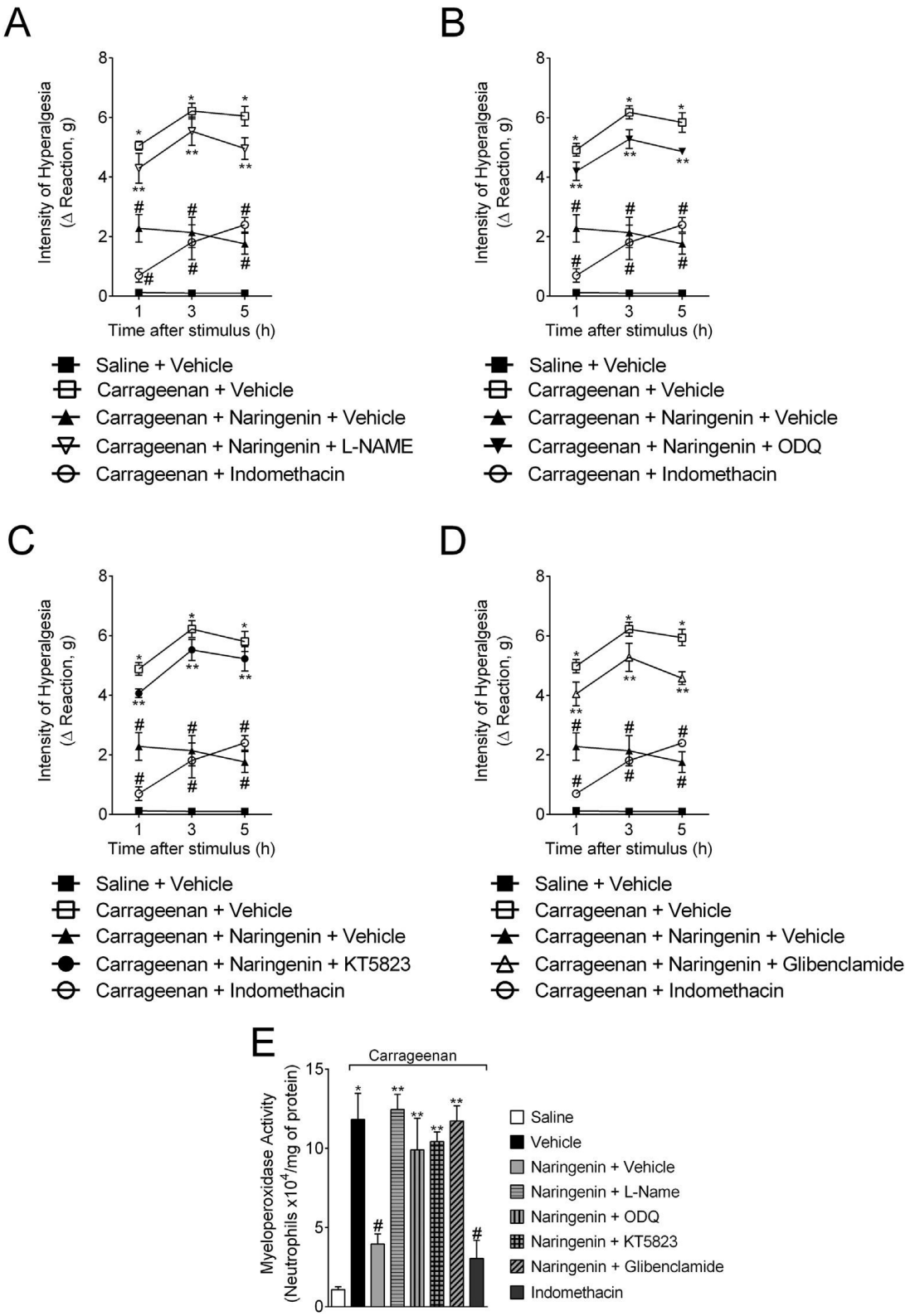

Fig. 6. Naringenin inhibits inflammatory pain and MPO activity by activating the NO−cyclic GMP−protein kinase G−ATP-sensitive K+ channel signaling pathway. Mice were treated with control drug indomethacin alone (5 mg/kg, i.p.) or l-NAME (A and E, NOS inhibitor; 90 mg/kg, i.p., diluted in saline, 60 min), ODQ (B and E, soluble guanylate cyclase inhibitor; 0.3 mg/kg, i.p., diluted in 2% DMSO in saline, 30 min), KT5823 (C and E, PKG inhibitor; 0.5 μg/mice, i.p., diluted in 2% DMSO in saline, 5 min), or glibenclamide (D and E, ATP-sensitive potassium channel blocker; 0.3 mg/kg, i.p., diluted in 5% Tween 80 in saline, 45 min) before treatment with naringenin (50 mg/kg, p.o.) and after an additional 30 min received carrageenan stimulus (300

μg/paw). Mechanical hyperalgesia was evaluated 1–5 h after carrageen injection (A–D). Paw skin samples were collected 3 h after stimulus and MPO activity was determined (E). Total protein was quantified and used to normalize the data from MPO test. Results are means ± SEM of six mice per group per experiment and are representative of two independent experiments [*p < 0.05 compared with the saline group, #p < 0.05 compared to carrageenan group, **p < 0.05 comparing the group treated with naringenin and the groups co-treated with naringenin and l-NAME (A and E), ODQ (B and E), KT5823 (C and E), and with glibenclamide (D and E) (Two-way ANOVA followed by Tukey's post hoc)].

Naringenin does not induce liver damage or gastric lesions. To evaluate whether naringenin induces liver damage or gastric lesions, plasma levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT), and stomach tissue myeloperoxidase (MPO) activity were measured. For both experimental procedures, the same experimental protocol was used. Mice were treated daily with naringenin (50 mg/kg, p.o.), indomethacin (positive control, 5 mg/kg, i.p., diluted in tris/HCl buffer), or vehicle (tris/HCl buffer, pH 8.0) for 7 days. Mice treated with naringenin or vehicle exhibited similar plasma AST and ALT levels (Fig. 7A and B), indicating that the compound does not induce liver damage. On the other hand, indomethacin treatment induced significant increases in AST and ALT plasma levels. The treatment with naringenin during seven-day did not alter MPO activity in stomach samples (Fig. 7C) compared to the vehicle-treated group, indicating that no gastric lesions occurred. On the other hand, indomethacin induced a significant increase of stomach MPO activity, indicating gastric lesions.

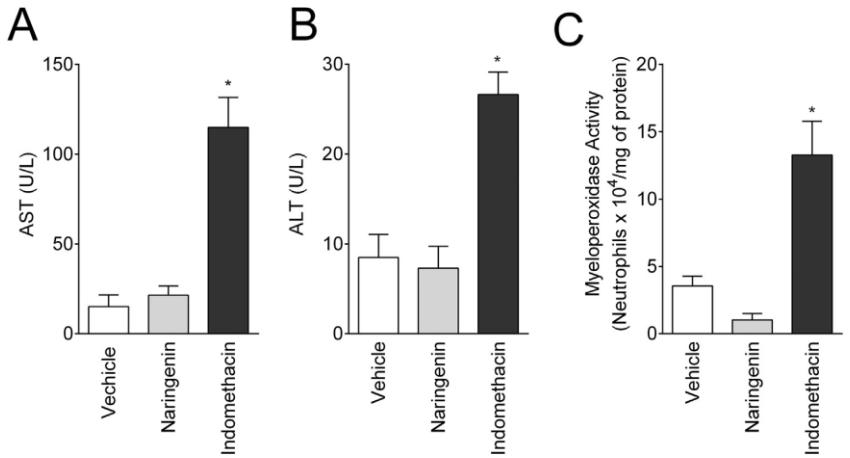

Fig. 7. Naringenin does not alter the plasma levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT), or induces gastric lesion. Mice received p.o. daily treatment with vehicle (saline or Tris/HCl buffer, pH 8.0), naringenin (50 mg/kg), or indomethacin (5 mg/kg). After seven days of treatment, mice were terminally anesthetized and blood was collected in heparinized microcentrifuge tubes for analysis of AST (A) and ALT (B) levels, and samples of the stomach were collected for MPO activity measurement (C) (n = 8). Total protein was quantified and used to normalize the data from MPO test. [*p < 0.05 compared with the vehicle or naringenin groups (one-way ANOVA followed by Tukey's post hoc).].

4. Discussion

Our results demonstrate that naringenin treatment reduces inflammatory pain by four main mechanisms: 1) inhibition of oxidative stress; 2) inhibition of NF-κB activation and hyperalgesic cytokine production; 3) activation of the NO- cyclic GMP- PKG- ATP sensitive K+ channel pathway; and 4) inhibition of neutrophil recruitment. Therefore, the present data highlight the analgesic potential of naringenin together with its safety since it did not present side effects common to non-steroidal anti-inflammatory drugs such as gastric ulcers and liver damage.

Pain is one of the clinical signs of inflammation and certainly the most unpleasant. Over the years, it has become clear that the contribution of oxidative stress is not only related to tissue damage, but rather represents cellular signaling pathways in physiopathology of several diseases. Focusing on pain, the inhibition of oxidative stress with antioxidants, superoxide dismutase mimetic and inhibitors of NADPH oxidase reduce inflammatory pain (Fattori et al., 2015, Khattab, 2006, Wang et al., 2004, Yamacita-Borin et al., 2015 and Zhao et al., 2014). In line with this, inducing oxidative stress and injecting ROS generators induce pain-like behavior (Fattori et al., 2015, Kim et al., 2008, Ndengele et al., 2008 and Wang et al., 2004). In cell free systems, naringenin inhibits lipoperoxidation and scavenges free radicals (Cavia-Saiz et al., 2010, Verri et al., 2012 and Yu et al., 2005). In fact, an analgesic dose of naringenin inhibited carrageenan-induced GSH depletion in vivo in the present study.

There is a very close relationship between oxidative stress and cytokine production. For instance, superoxide anion induces pain-like behavior dependent on TNF-α/TNFR1 signaling and a superoxide dismutase mimetic and NADPH oxidase inhibitor reduce TNF-α-induced hyperalgesia (Yamacita-Borin et al., 2015). This interaction between oxidative stress and cytokines is in part dependent on the transcription factor NF-κB, which can be activated by oxidative stress and cytokines (Jamaluddin et al., 2007 and Verri et al., 2006). In agreement, naringenin inhibited carrageenan-induced NF-κB activation. Inhibiting NF-κB diminishes the production of IL-33, TNF-α and IL-1β in vivo and in vitro (Ruiz-Miyazawa et al., 2015). In the present study, naringenin inhibited carrageenan-induced production of the cytokines IL-33, TNF-α, and IL-1β. After carrageenan injection a cascade of cytokines initiates with IL-33 that triggers the production of TNF-α, which in turn induces IL-1β production (Zarpelon et al., 2013). IL-33, TNF-α, and IL-1β act indirectly to induce hyperalgesia by triggering the

production of prostanoids such as PGE_2, which sensitizes the nociceptors (Zarpelon et al., 2013). There is also evidence that TNF-α and IL-1β sensitize nociceptors and enhance synaptic transmission to induce pain (Binshtok et al., 2008, Gruber-Schoffnegger et al., 2013, Kawasaki et al., 2008 and Zarpelon et al., 2016). To our knowledge, this is the first evidence that naringenin inhibits carrageenan-induced NF-κB activation in vivo. And although this mechanism has been shown in other models in vivo and in vitro, the specific target of naringenin in NF-κB pathway is still unknown (Dou et al., 2013, Park et al., 2012 and Raza et al., 2013). It is possible that naringenin inhibits NF-κB by acting as a Nrf2 activator (Lou et al., 2014). Nrf2 improves cellular antioxidant capacity and counteracts NF-κB signaling without inducing immunosuppression (Kim et al., 2010). In an inverse pathway, ROS induce NF-κB activation since this transcription factor is also regulated by cellular redox state. This evidence is supported by antioxidant prevention of oxidative stress-induced NF-κB activation (Jamaluddin et al., 2007 and Verri et al., 2012). In agreement with that, we observed that naringenin treatment prevented carrageenan-induced reduction of GSH concentration, maintaining GSH at its baseline levels. GSH is a downstream target of Nfr2 (Kim et al., 2010). Considering GSH concentration is an indicative of the redox state of cells and tissues, our results suggest that naringenin inhibited the oxidative stress triggered by carrageenan and, consequently, this could be a contributing mechanism to naringenin inhibition of carrageenan-induced activation of NF-κB.

Naringenin inhibited PGE_2-induced mechanical hyperalgesia, suggesting possible neuronal effects would be involved in its mechanism of action since PGE_2 directly sensitizes the nociceptive neuron (Cunha et al., 2012). The control drug dipyrone also inhibited PGE_2-induced mechanical hyperalgesia, which strength the role of naringenin over nociceptors since dipyrone reduces hyperalgesia by acting in TRPA1 and activating the NO-cyclic GMP-PKG-ATP sensitive K^+ channel pathway (Alves and Duarte, 2002, Nassini et al., 2015 and Sachs et al., 2004). To our knowledge, this is the first demonstration that naringenin inhibits inflammatory mechanical hyperalgesia by activating the analgesic NO-cyclic GMP-PKG-ATP sensitive K^+ channel pathway. Neuronal nitric oxide synthase produces NO, which in turn activates the cyclic GMP-PKG-ATP sensitive K^+ channel pathway at the dorsal root ganglia and spinal cord levels inducing analgesia (Maihofner et al., 2000, Schmidtko et al., 2009 and Shi et al., 1998). Triggering the NO-cyclic GMP-PKG-ATP sensitive K+ channel is also an important analgesic mechanism of opioids, some non-steroidal anti-inflammatory drugs such as diclofenac and dipyrone, natural products and NO donors (Cunha et al., 2012,

Possebon et al., 2014, Sachs et al., 2004, Santodomingo-Garzon et al., 2006 and Staurengo-Ferrari et al., 2014). Furthermore, the inhibitory effect of naringenin on neutrophil recruitment also depends on the activation of the NO-cyclic GMP-PKG-ATP sensitive K+ channel pathway as observed by the MPO activity. This is in agreement with the inhibitory effect of vascular endothelial NO over adhesion molecules expression by activating the cyclic GMP-PKG-ATP sensitive K+ channel pathway, resulting in reduced neutrophil recruitment (Dal Secco et al., 2006, Paula-Neto et al., 2011 and Staurengo-Ferrari et al., 2014). The inhibition of neutrophil recruitment to the paw skin might also contribute to the analgesic mechanism of naringenin since neutrophils produce nociceptive molecules in the inflammatory foci (Guerrero et al., 2008, Ting et al., 2008 and Verri et al., 2009). Importantly, the naringenin reduction of IL-33, TNF-α and IL-1β production in the paw skin is also a contributing mechanism to the observed reduction of MPO activity since these cytokines chemoattract neutrophils (Verri et al., 2010).

Naringenin blocks TRPM3 (transient receptor potential melastatin-3) (Straub et al., 2013), a channel necessary to the perception of noxious heat (Vriens et al., 2011). Moreover, naringenin does not affect TRPM1 (transient receptor potential melastatin-1) and TRPA1 (transient receptor potential ankyrin 1), and reduces TRPV1 activation only at high concentrations (Straub et al., 2013). Nevertheless, naringenin inhibited acetic acid-, capsaicin- and formalin-induced overt pain-like behavior, which depends, at least in part, on TRPV1, TRPV1, and TRPA1, respectively (Caterina et al., 1997, Liu et al., 2013 and McNamara et al., 2007). Furthermore, naringenin is a TRPM8 agonist (Straub et al., 2013). Treatment with TRPM8 agonist partially inhibits the overt pain-like behavior induced by capsaicin (TRPV1 agonist), noxious heat (activates TRPV1), acrolein (TRPA1 agonist) and acetic acid (depends on TRPV1 activation and inflammatory cytokines); and the mechanical hyperalgesia induced by CFA (dependent on hyperalgesic cytokines and NF-κB) (Caterina et al., 1997, Liu et al., 2013, Magro et al., 2013, McNamara et al., 2007, Verri et al., 2008 and Woolf et al., 1997). Therefore, the TRPM8 agonism might be a contributing mechanism of naringenin analgesia. Additionally, the analgesic mechanisms of naringenin might not solely depend on targeting TRP channels. In this sense, naringenin reduces the release of neuropeptides that act peripherally to increase pain and inflammation such as substance P (Gilligan et al., 1994 and Nakagawa et al., 1995). Importantly, there is significant participation of inflammatory mediators in all models tested in the present study (Caterina et al., 1997, Cunha et al., 2005, McNamara et al., 2007, Verri et al., 2006 and Woolf et al., 1997), which supports the

importance of the inhibition of NF-κB activation and cytokine production for the naringenin analgesic effect. Moreover, the pain-like behavior in these models is susceptible to activation of the NO-cyclic GMP-PKG-ATP sensitive K+ channel pathway (Staurengo-Ferrari et al., 2014, Steiner et al., 2001 and Zarpelon et al., 2013).

5. Conclusions

The flavonoid naringenin presents analgesic effects in varied models, which supports its potential as an analgesic molecule. Importantly, naringenin did not induce common side effects observed with non-steroidal anti-inflammatory drugs in a 7 days treatment protocol. The mechanisms of naringenin involve the blockade of TRPM3, partial blockade of TRPV1 and TRPM8 agonism (Straub et al., 2013) as previously shown. In the present study, we add evidence that the analgesic mechanisms of naringenin also involve the inhibition of oxidative stress, NF-κB activation, hyperalgesic cytokine production, and neutrophil recruitment as well as activation of the NO-cyclic GMP-PKG-ATP sensitive K+ channel pathway.

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The citrus flavonone naringenin reduces lipopolysaccharide-induced inflammatory pain and leukocyte recruitment by inhibiting NF-κB activation ☆

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Abstract

Lipopolysaccharide (LPS) is the major structural component of Gram-negative bacteria cell wall and a highly pro-inflammatory toxin. Naringenin is found in Citrus fruits and exhibits antioxidant and anti-inflammatory properties through inhibition of NF-κB activation but its effects in LPS-induced inflammatory pain and leukocyte recruitment were not investigated yet. We investigated the effects of naringenin in mechanical hyperalgesia, thermal hyperalgesia and leukocyte recruitment induced by intraplantar injection of LPS in mice. We found that naringenin reduced hyperalgesia to mechanical and thermal stimuli, myeloperoxidase (MPO, a neutrophil and macrophage marker) and N-acetyl-β-D-glucosaminidase (NAG, a macrophage marker) activities, oxidative stress and cytokine (TNF-α, IL-1β, IL-6, and IL-12) production in the paw skin. In the peritoneal cavity, naringenin reduced neutrophil and mononuclear cell recruitment, and abrogated MPO and NAG activity, cytokine and superoxide anion production, and lipid peroxidation. In vitro, pre-treatment with naringenin inhibited superoxide anion and cytokine (TNF-α, IL-1β, IL-6, and IL-12) production by LPS-stimulated RAW 264.7 macrophages. Finally, we demonstrated that naringenin inhibited NF-κB activation in vitro and in vivo. Therefore, naringenin is a promising compound to treat LPS-induced inflammatory pain and leukocyte recruitment.

Keywords
Inflammatory pain; Cytokines; Oxidative stress; Leukocyte recruitment; NF-κB

1. Introduction

Naringenin (NGN; 4′,5,7-thrihydroxyflavanone) is a flavonone that occurs naturally in vegetables, fruits, herbs and nuts widely consumed by humans, including *Lycopersicum esculentum* (tomato) [1], *Citrus paradise* (grapefruit) [2], *Citrus sinensis* (orange) [3] and [4], *Lippia graveolens* (mexican oregano) [5] and nuts like pistachio [6]. An increasing number of pre-clinical studies suggest that NGN presents great potential to treat a wide range of pathological conditions that are related to inflammation and oxidative stress, including ultraviolet B-induced skin damage [7], daunorubicin-induced nephrotoxicity [8], diabethic

neuropathy [9], and neuropathic pain [10] and [11]. Its anti-inflammatory activity is of great interest due to the role of inflammatory mediators in diseases that significantly reduce patients' quality of life, mainly because of inflammatory pain.

Pain is a clinical sign of inflammation and an important complaint of patients with infectious diseases [12], [13] and [14]. Lipopolysaccharide (LPS), the major constituent of gram-negative bacterial cell walls, is released in large amounts during infection by gram-negative bacteria due to bacteriolytic activity of mediators produced by the immune system. Neutrophils represent important sources of these and other mediators that, despite killing of bacteria, increase the release of LPS that can lead to additional postinfectious sequelae or even to endotoxic shock [15]. Additionally, LPS and inflammatory mediators act synergistically to increase pain sensitivity [16], [17] and [18]. Therefore, targeting the effects of LPS is of major clinical importance. NGN inhibits the synthesis of inflammatory mediators by macrophages infected with a gram-negative bacteria [19] and kills gram-positive bacteria [20], but the efficacy of naringenin in reducing LPS-induced pain and leukocyte recruitment were not described previously. Therefore, we aimed to evaluate the effects of NGN in LPS-induced inflammatory pain, leukocyte recruitment in mice and its mechanisms of action.

2. Materials and methods

2.1. General In Vivo experimental procedures

Mice were treated by oral (p.o.) gavage with naringenin (NGN; 16.7, 50, or 150 mg/kg, 100 μL) or vehicle (sterile saline, 100 μL) 1 h before LPS injection. Hyperalgesia to mechanical and thermal stimuli was evaluated 1–5 h after intraplantar (i.pl.) injection with 100 ng of LPS. Neutrophil and macrophage recruitment to the plantar tissue was evaluated 5 h after LPS injection by myeloperoxidase (MPO) and by N-acetyl-β-D-glucosaminidase (NAG) assays, respectively. Oxidative stress, cytokine production, and NF-κB activity in paw skin tissue were determined 3 h (peak of hyperalgesia) after LPS i.pl. injection. In another approach, LPS (100 ng) was administered by intraperitoneal (i.p.) injection. Leukocyte migration to the peritoneal cavity, oxidative stress, and cytokine levels were evaluated 4 h after LPS i.p. injection.

2.2. Test compounds

The compounds used in this study were saline (NaCl 0,9%; Fresenius Kabi Brasil Ltda. Aquiraz, CE, Brazil), naringenin (≥ 95% purity, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and LPS (lipopolysaccharides from E. coli, Santa Cruz Biotechnology, Santa Cruz, CA, United States). Immediately before the p.o. administration, naringenin was diluted in saline solution.

2.3. Animals

Male Swiss mice (25–30 g) from the Universidade Estadual de Londrina, Paraná, Brazil, were used in this study. Mice were housed in standard clear plastic cages with free access to food and water and a light/dark cycle of 12–12 h at 21 °C. All behavioral testing was performed between 9 a.m. and 5 p.m. in a temperature-controlled (21 °C) room. Animal care and handling procedures were approved by the Ethics Committee of the Universidade Estadual de Londrina (process number 32.186.2012.64). All efforts were made to minimize animals' suffering and to reduce the number of animals used.

2.4. Mechanical hyperalgesia test

Mechanical hyperalgesia was measured by an electronic version of von Frey filaments [21]. The test consisted of evoking a hind paw reflex with a hand-held force transducer (electronic anesthesiometer; Insight Equipamentos, Ribeirao Preto, SP, Brazil). After paw withdrawal, the pressure intensity was recorded automatically, and the values were an average of three measurements. Mice were tested before (basal) and after stimulus injection. The results are expressed as delta (Δ) withdrawal threshold (in g), calculated by subtracting the mean measurements obtained at 1, 3, or 5 h after i.pl. stimulus with LPS from the basal mean measurements.

2.5. Hot plate test

Mice were placed on a hot plate apparatus (Hot Plate HP-2002, Insight Equipamentos, Ribeirao Preto, SP, Brazil) maintained at 55 °C. The paw withdrawal reaction was registered. A maximum latency (cut-off) was set at 20 s to avoid tissue damage [22] and [23].

2.6. MPO activity

The neutrophil recruitment to the paw tissue was evaluated by the MPO kinetic-colorimetric assay [24] and [25]. Samples were homogenized using a tissue-tearor (Biospec®) in ice-cold K_2HPO_4 buffer (400 μL, 50 mM, pH 6.0) containing HTAB (0.5% weight/volume), and the homogenates were centrifuged (16,100 g × 2 min × 4 °C). The supernatants (30 μL) were mixed with K_2HPO_4 buffer (200 μL, 50 mM, pH 6.0) containing o-dianisidine dihydrochloride (0.0167%, w/v) and hydrogen peroxide (0.015%, v/v). The absorbance was determined after 5 min at 450 nm (Multiskan GO Microplate Spectrophotometer, Thermo Scientific, Vantaa, Finland). The results of MPO activity are expressed as the number of neutrophils per mg of tissue by using a standard curve of neutrophils (196–400,000 cells).

2.7. NAG activity

NAG (N-acetyl-β-D-glucosaminidase) activity was determined by an adapted colorimetric method previously described [26]. Briefly, the supernatants (20 μL), obtained in the MPO activity assay, were placed in a 96-well plate and mixed with K_2HPO_4 buffer (80 μL, 50 mM, pH 6.0). The reaction was initiated by the addition of K_2HPO_4 buffer (100 μL, 50 mM, pH 6.0) containing 4-nitrophenyl N-acetyl-β-D-glucosaminide substrate (2.24 mM). The plate was incubated at 37 °C for 10 min, and glycine buffer (100 μL, 0.2 M pH 10.6) was added. The enzymatic activity was determined spectrophotometrically at 400 nm (Multiskan GO ThermoScientific). The results of NAG activity are expressed as the number of macrophages per mg of tissue by using a standard curve of macrophages (196–400,000 cells).

2.8. ABTS and FRAP assays

The ability of samples to resist oxidative damage was determined by its free radical scavenging (ABTS [2,2′-Azinobis-3-ethylbenzothiazoline 6-sulfonic acid] assay) and ferric reducing (FRAP assay) properties. The tests were adapted to a 96-well microplate format as previously described [26]. Plantar tissue samples were collected 3 h after i.pl. stimulus with LPS and homogenized immediately in ice-cold KCl buffer (500 μL, 1.15% w/v). The absorbance of ABTS and FRAP assays were measured at 730 and 595 nm (Multiskan GO Thermo Scientific), respectively, and the results were equated against a standard Trolox curve (0.02–20 nmol).

2.9. Superoxide anion production

The measurement of superoxide anion production in tissue homogenates (10 mg/mL in 1.15% KCl) was performed using the nitroblue tetrazolium (NBT) assay adapted to a microplate as described previously [27]. The NBT reduction was measured at 600 nm (Multiskan GO, Thermo Scientific). The tissue weight was used for data normalization.

2.10. Lipid peroxidation

Lipid peroxidation was measured by the Thiobarbituric Acid Reactive Substances (TBARS) assay as described previously [28]. Plantar tissue samples were collected 3 h after i.pl. stimulus with LPS and homogenized immediately in ice-cold KCl buffer (500 μL, 1.15% w/v). Malondialdehyde (MDA) levels, an intermediate product of lipid peroxidation, was determined in samples by the difference between absorbance at 535 and 572 nm (Multiskan GO, Thermo Scientific). Results are reported as nmol of MDA per mg of tissue.

2.11. Enzyme-linked immunosorbent assays (ELISA)

Mice were terminally anesthetized 3 h after i.pl. stimulus, and samples from paw skin were collected. ELISA was performed to evaluate p65 activation by the ratio of total NF-κB p65/phospho-NF-κB p65 Ser536 (Cell Signaling Technology, Danvers, MA, USA) and cytokine production (TNF-α, IL-1β, IL-6 and IL-12) (eBioscience, San Diego, CA, USA) following the manufacturers instructions.

2.12. Cell culture, luciferase activity and lactate dehydrogenase (LDH) leakage

The murine macrophage cell line RAW 264.7, stably expressing luciferase on NF-κB responsive promoter (pNF-κB-Luc) [29] and [30], was routinely cultured in Dulbecco's modified Eagle's medium supplemented (10% fetal bovine serum and penicillin–streptomycin) at 37 °C in a humidified atmosphere of 5% CO_2. For the luciferase reporter assay, RAW 264.7 macrophages (3 × 105 cells/well) were grown in 24 well plates. After culturing over-night, cells were treated with different drug concentrations and stimulated with lipopolysaccharide (1 μg/mL). Intracellular contents were extracted in lysis buffer (TNT), and the luciferase activity in cell lysates was determined on a luminometer (Victor X5, PerkinElmer, Waltham, MA) using the Dual Luciferase Reporter assay system (Promega, Wisconsin, USA). Data are expressed as a ratio of relative luminescence units (RLU). LDH leakage was measured as described previously [29] and [30] using supernatants to determine cytotoxicity by Cytotoxicity Detection KitPLUS (Roche Applied Science, Mannheim, Germany) according to the manufacturer’s directions.

2.13. Data analyses

Results are means ± SEM of 6 mice per group per experiment, each experiment was performed twice. The in vitro experiments are means ± SEM of three independent experiments performed in triplicate. Two-way ANOVA was used to compare the groups and doses at all times when the parameters were measured at different times after the stimulus injection. The analyzed factors were treatments, time and time versus treatment interaction.

One-way ANOVA followed by Tukey's test was performed for each time-point. $P < .05$ was considered significant.

3. Results

3.1. Naringenin (NGN) inhibits LPS-induced inflammatory pain

Mice were treated with NGN (16.7–150 mg/kg, p.o.) 1 h before i.pl. injection with LPS. Treatment with NGN in the doses of 50 and 150 mg/kg inhibited hyperalgesia induced by LPS to mechanical (Fig. 1A) and thermal stimuli (Fig. 1B). No effects were observed at the dose of 16.7 mg/kg of NGN. LPS also induced neutrophil (MPO activity) (Fig. 1C) and macrophage (MPO and NAG activity) (Fig. 1D) recruitment in the paw skin, while NGN inhibited these effects (Fig. 1C–1D) at the doses of 50 and 150 mg/kg with no statistically significant differences between these two doses. Thus, we chose to use the dose of 50 mg/kg was in the following experiments.

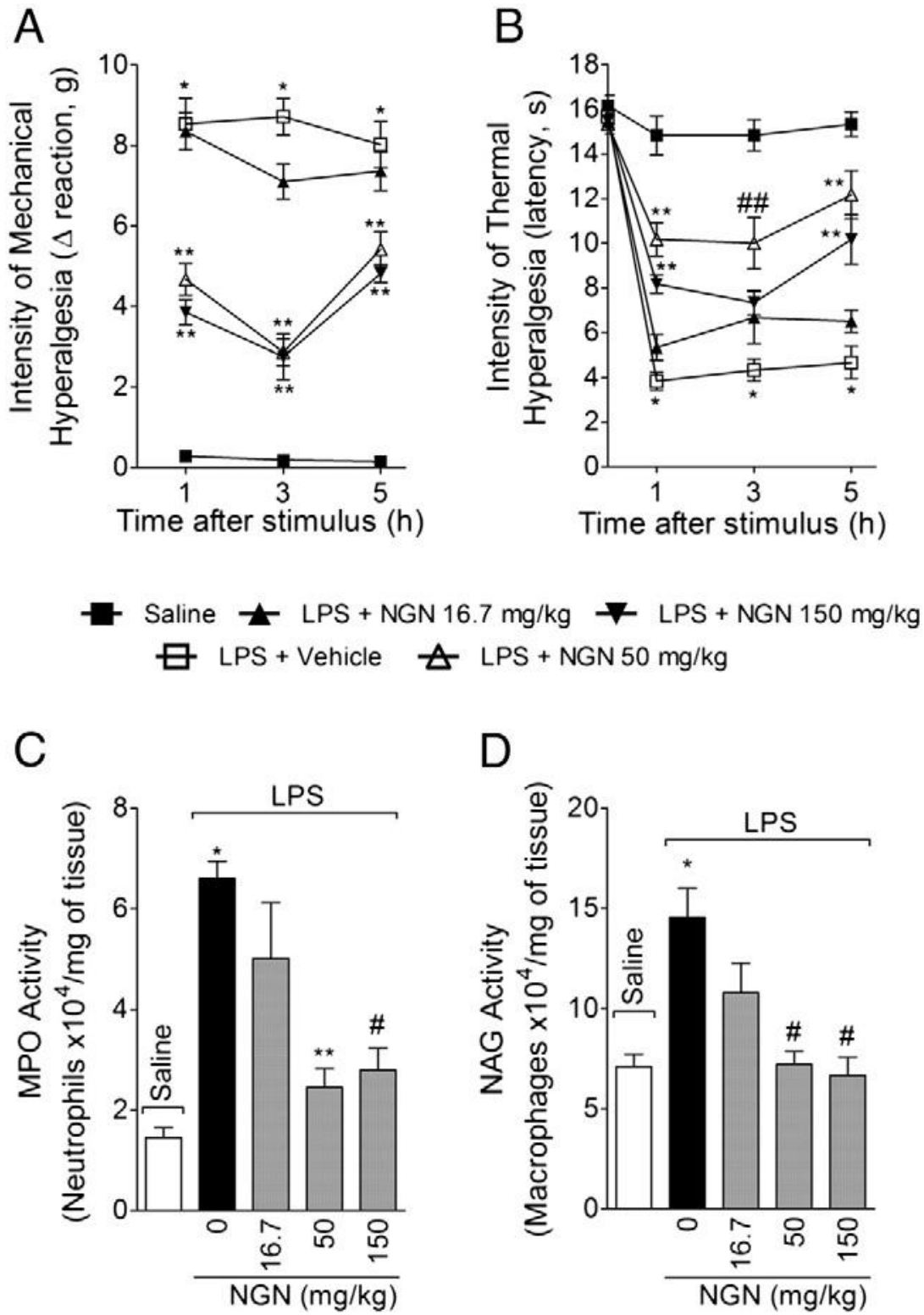

Fig. 1. Naringenin (NGN) inhibits LPS-induced hyperalgesia and leukocyte recruitment in a dose-dependent manner. (A) Mechanical and (B) thermal hyperalgesia were measured 1–5 h after LPS injection. (C) MPO and (D) NAG activity were assessed in paw skin tissues 5 h after LPS injection. Results are means ± SEM, n = 6 mice per group per experiment, two independent experiments (*P < .05 vs. saline group; #P < .05 vs. 0 mg/kg group, ANOVA followed by Tukey's test).

3.2. NGN inhibits oxidative stress induced by LPS

Neutrophils and macrophages are important sources of reactive oxygen species that contribute to inflammatory pain [31], [32], [33], [34], [35] and [36]. Therefore, we next evaluated the antioxidant status of paw skin tissue 3 h after LPS i.pl. stimulus and the effects of NGN treatment (50 mg/kg, p.o., 1 h before) in this system. LPS reduced ABTS radical scavenging and the ferric reducing (FRAP) abilities of paw skin tissue (Fig. 2A–B). Furthermore, treatment with NGN inhibited LPS-induced oxidative stress (Fig. 2A–B).

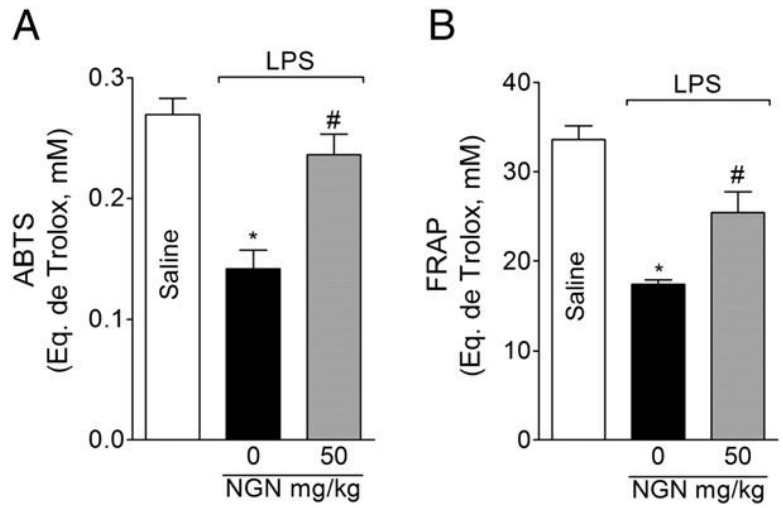

Fig. 2. Effects of naringenin (NGN) in LPS-induced oxidative stress in paw skin tissue. (A) Free radical scavenging ability (ABTS assay) and (B) the ferric reducing antioxidant power (FRAP assay) were compared with a Trolox curve. Samples were collected 3 h after LPS injection. Results are means ± SEM, n = 6 mice per group per experiment, two independent experiments (*P < .05 vs. saline group; #P < .05 vs. 0 mg/kg group, ANOVA followed by Tukey's test).

3.3. NGN reduces LPS-induced cytokine production in the paw skin

Mice received NGN (50 mg/kg, p.o., 1 h before LPS), and paw skin concentrations of pro-inflammatory/hyperalgesic cytokines TNF-α, IL-1β, IL-6, and IL-12 were determined 3 h after LPS i.pl. injection. LPS injection induced the production of all evaluated cytokines (Fig. 3). On the other hand, NGN treatment (50 mg/kg) reduced significantly the LPS-induced production of TNF-α, IL-1β, IL-6, and IL-12 (Fig. 3).

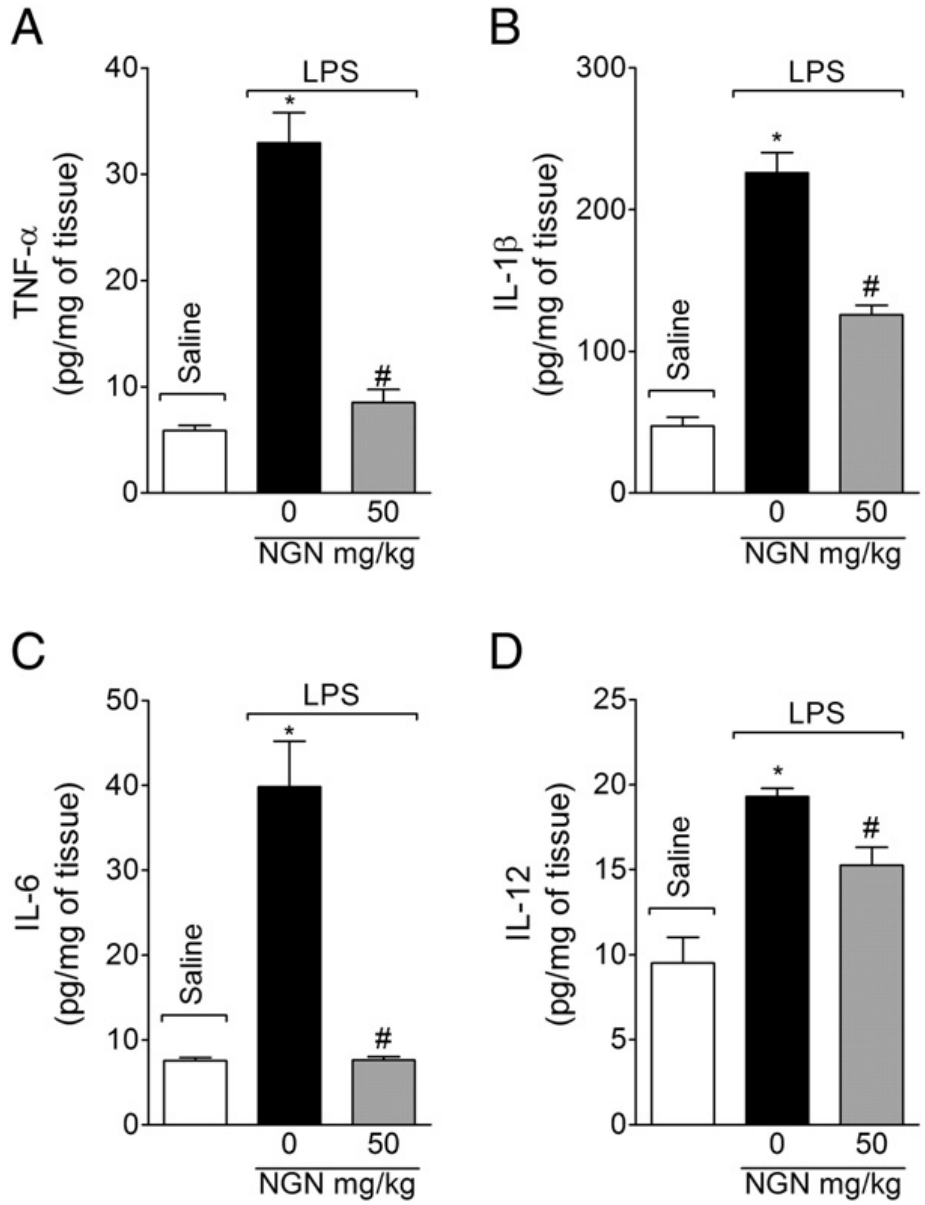

Fig. 3. Naringenin (NGN) inhibits LPS-induced production of hyperalgesic cytokines in paw skin tissue. Samples of the paw skin tissue were collected 3 h after LPS injection and the levels of TNF-α (A), IL-1β (B), IL-6 (C), and IL-12 (D) were determined by ELISA. Results are means ± SEM, n = 6 mice per group per experiment, two independent experiments [*P < .05 vs. saline control; #P < .05 vs. 0 mg/kg (vehicle), (ANOVA followed by Tukey's test)].

3.4. NGN reduces leukocyte recruitment, superoxide anion production, lipid peroxidation, and cytokine production induced by LPS in the peritoneal cavity

Mice were treated with NGN (16.7–150 mg/kg, p.o.) 1 h before peritonitis induced by intraperitoneal (i.p.) injection of LPS (100 ng). Peritoneal lavage fluid was harvested 5 h after LPS injection. LPS induced leukocyte recruitment into the peritoneal cavity (Fig. 4A–C).

Treatment with naringenin reduced total leukocyte (Fig. 4A), neutrophil (Fig. 4B) and mononuclear cell (Fig. 4C) recruitment to the peritoneal cavity at the doses of 50 and 100 mg/kg. Furthermore, NGN abrogated LPS-induced increases in MPO (Fig. 4D) and NAG (Fig. 4E) activities, which lines up well with the results obtained in the paw skin (Fig. 1). Treatment with NGN (50 mg/kg) inhibited LPS-induced superoxide anion production (NBT assay) (Fig. 5A) and malondialdehyde (MDA) formation, a product of lipid peroxidation (Fig. 5B). Moreover, LPS injection increased TNF-α, IL-1β, IL-6, and IL-12 concentration in the peritoneal cavity, and NGN (50 mg/kg) treatment lowered the concentration of all these cytokines (Fig. 6). These data corroborate that NGN modulates immune responses by inhibiting leukocyte activation and recruitment.

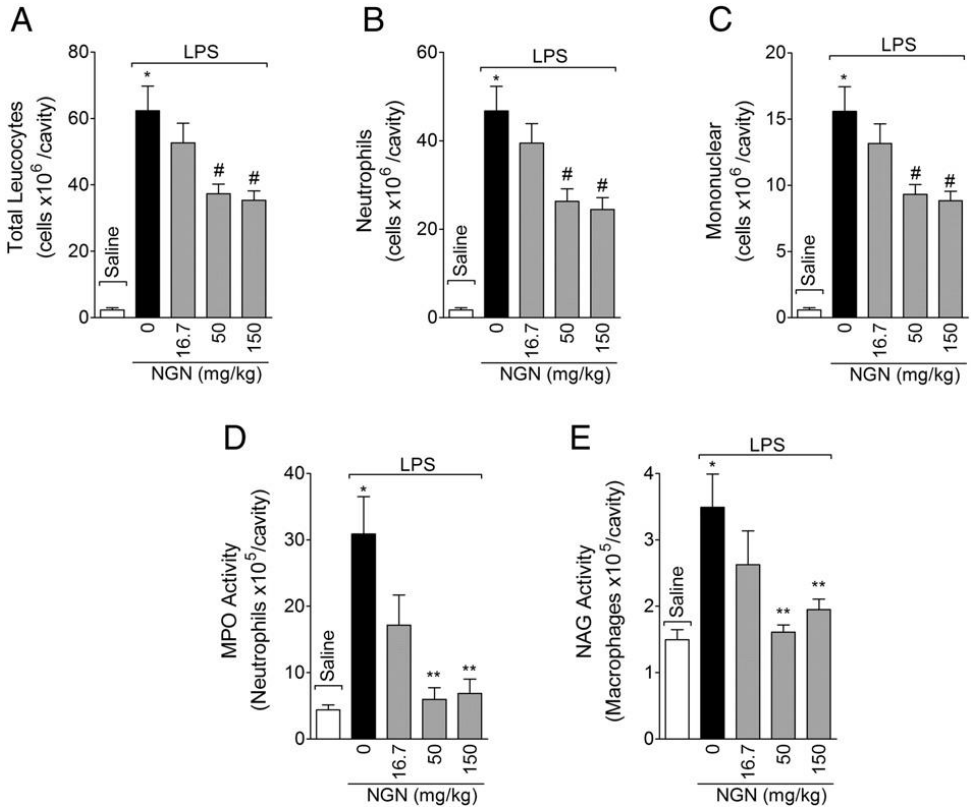

Fig. 4. Treatment with naringenin (NGN) inhibits LPS-induced leukocyte recruitment to the peritoneal cavity in a dose-dependent manner. Peritoneal cells were harvested 6 h after LPS injection. Total (A) and differential (B and C) cell counts were performed with a cell counter and by Rosenfeld method, respectively. MPO (D) and NAG (E) activity were determined by colorimetric assays. Results are means ± SEM, n = 6 mice per group per experiment, two independent experiments [*P < .05 vs. saline control; #P < .05 vs. 0 mg/kg (vehicle), (ANOVA followed by Tukey's test)].

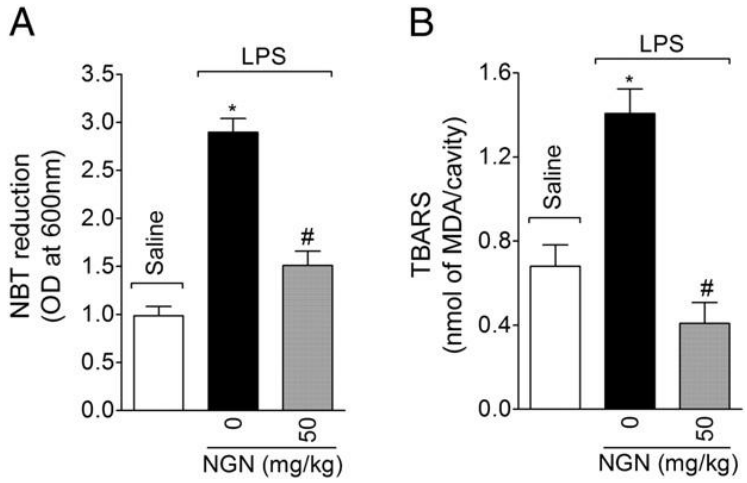

Fig. 5. Naringenin (NGN) inhibits LPS-induced superoxide production and lipid peroxidation in the peritoneal cavity. Peritoneal exudate was collected 6 h after LPS injection. Superoxide anion production (A) and lipid peroxidation (B) were determined by NTB and TBARS assays, respectively. Results are means ± SEM, n = 6 mice per group per experiment, two independent experiments [*P < .05 vs. saline control; #P < .05 vs. 0 mg/kg (vehicle), (ANOVA followed by Tukey's test)].

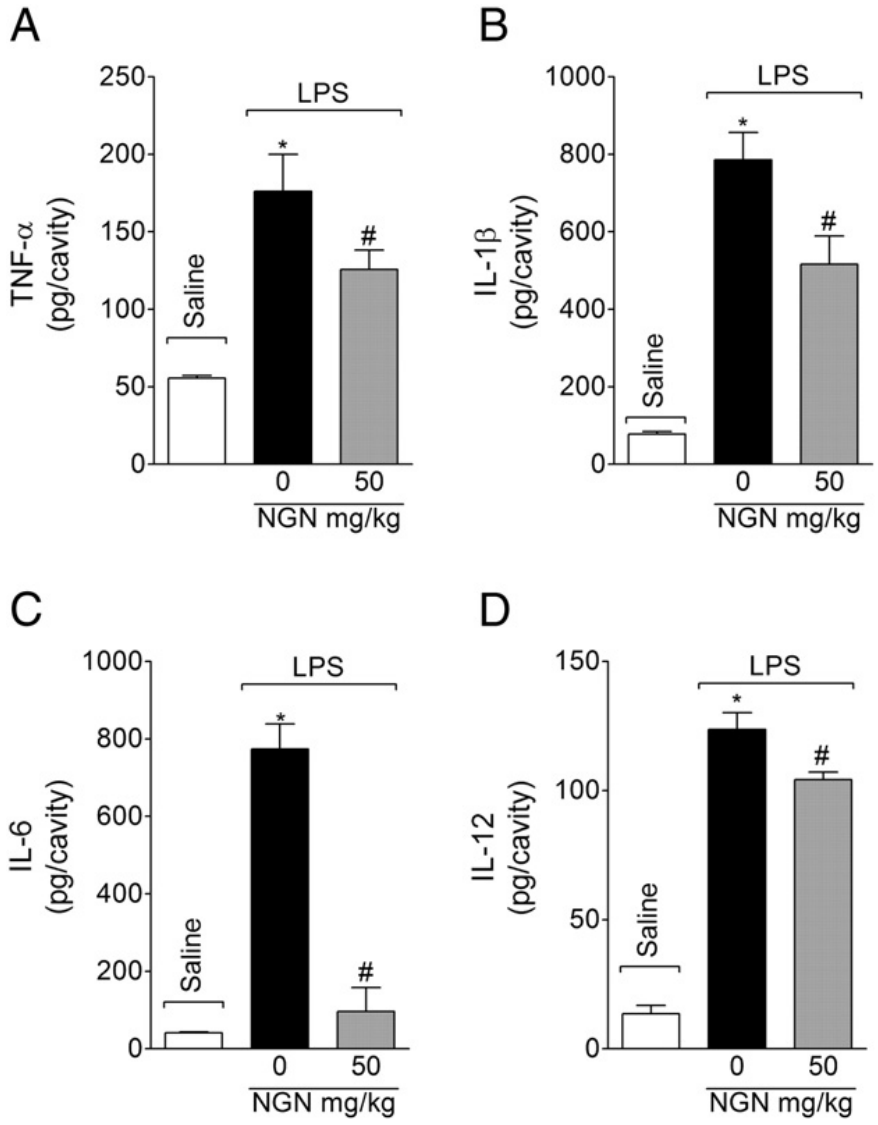

Fig. 6. Naringenin (NGN) inhibits LPS-induced cytokine production in peritoneal cavity. Peritoneal exudate was collected 6 h after LPS injection, and the levels of TNF-α (A), IL-1β (B), IL-6 (C), and IL-12 (D) were determined by ELISA. Results are means ± SEM, n = 6 mice per group per experiment, two independent experiments [*P < .05 vs. saline control; #P < .05 vs. 0 mg/kg (vehicle), (ANOVA followed by Tukey's test)].

3.5. NGN reduces NF-κB activation, cytokine production, and superoxide anion production by RAW 264.7 macrophages stimulated with LPS

LPS induces the expression of pro-inflammatory cytokines in a NF-κB-dependent manner. Considering this, we hypothesized that NGN may affect NF- κB signaling. The effects of NGN were evaluated in LPS-stimulated RAW 264.7 macrophages stably expressing luciferase under a NF-κB responsive promoter. Cells were pre-treated with NGN (30–1000 nM) 1 h before LPS stimulus (1 μg/mL). Supernatants were collected 6 h after LPS stimulus

and cell lysates were used to evaluate NF-κB activation by a luciferase activity assay (Fig. 7A). Additionally, the production of superoxide anion (Fig. 7B) and cytokines (Fig. 7C-F), as well as LDH concentration/cell viability (Fig. 7G) were also evaluated. LPS-induced NF-κB activation was reduced by NGN treatment at 300 and 1000 nM (Fig. 7A). Moreover, NGN reduced LPS-induced superoxide anions (Fig. 7B), TNF-α (Fig. 7C), IL-1β (Fig. 7D), IL-6 (Fig. 7E), and IL-12 secretion (Fig. 7F). These inhibitory effects showed a similar profile as for NF-κB activation inhibition (Fig. 7A), except that NGN inhibited LPS-induced superoxide anion production only at the concentration of 1000 nM (Fig. 7B) and the concentrations of 30 and 100 nM also inhibited IL-12 production (Fig. 7F). No cytotoxicity was induced by treatments (Fig. 7G). Therefore, we next evaluated whether NGN could also inhibit NF-κB activity in the paw skin.

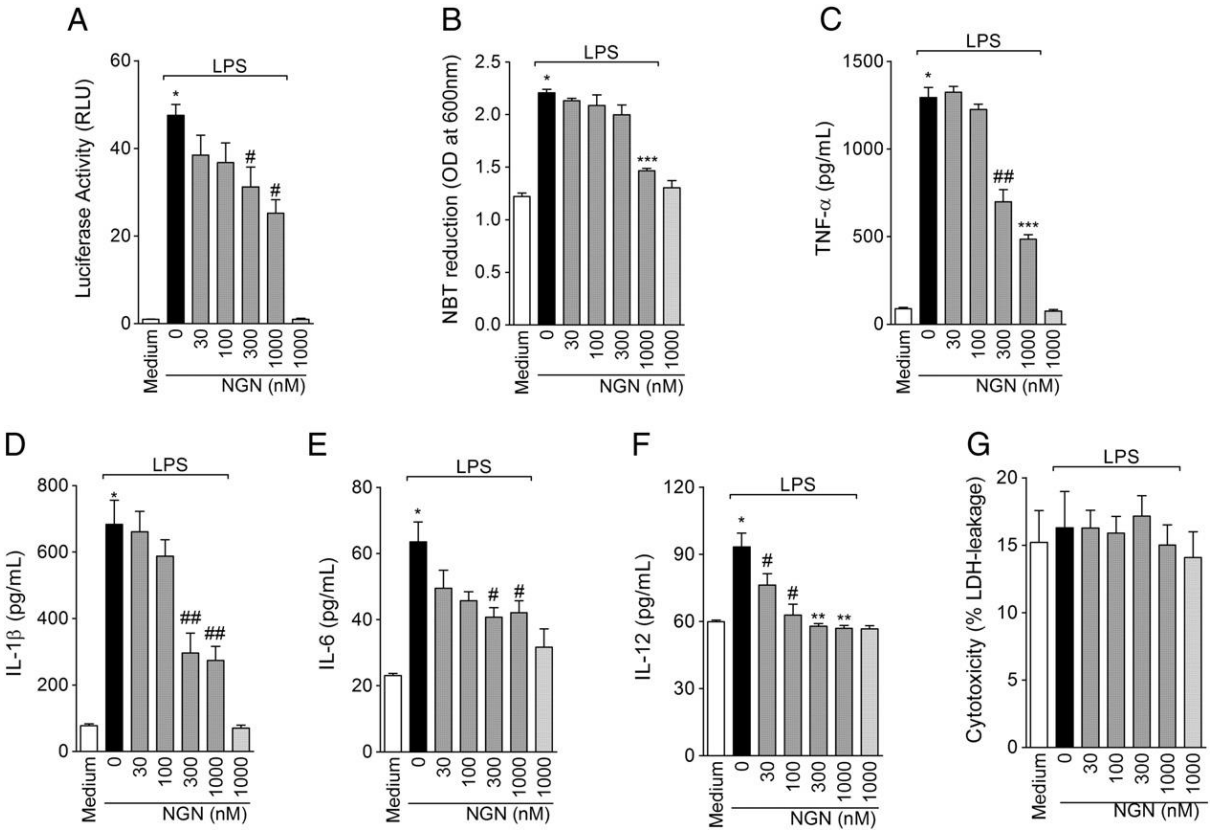

Fig. 7. Effect of naringenin (NGN) on LPS-induced NF-κB promoter activity in RAW 264.7 macrophage cell line. RAW 264.7 cells transfected with pNF-κB-Luc were incubated for 30 min with NGN (30–1000 nM) or vehicle (medium), and then stimulated with LPS (1 μg/ml) for 6 h. Then, supernatants were removed and NF-κB-dependent luciferase activity was measured in cell lysate (A). The levels of superoxide anion (B), cytokines (C-F), and lactate dehydrogenase (G) were determined in culture supernatants. Results are means ± SEM of three independent experiments performed in triplicate. RLU, relative luminescence units of fold change compared with medium control group [*P < .05 vs. medium control; #P < .05 vs. 0 nM (vehicle), (ANOVA followed by Tukey's test)].

3.6. NGN inhibits LPS-induced NF-κB activation in vivo

NF-κB activity in the paw skin was then investigated by measuring the ratio of total and phosphorylated NF-κB subunit p65. Mice were treated with 50 mg/kg of NGN 1 h before i.pl. injection of LPS (100 ng), and paw skin tissue samples were collected 3 h after stimulus. The concentration of total and phosphorylated NF-κB p65 levels were measured by ELISA. Corroborating the results of Fig. 7, NGN inhibited LPS-induced NF-κB activation in the paw skin as observed by a reversal of LPS-induced decreases in the ratio of total NF-κB/phosphorylated NF-κB (Fig. 8). The same dose of NGN also reduced inflammatory pain (Fig. 1), leukocyte recruitment (Fig. 1 and Fig. 4), oxidative stress (Fig. 2 and Fig. 5), and cytokine production (Fig. 3 and Fig. 6) in the paw skin and peritoneal cavity.

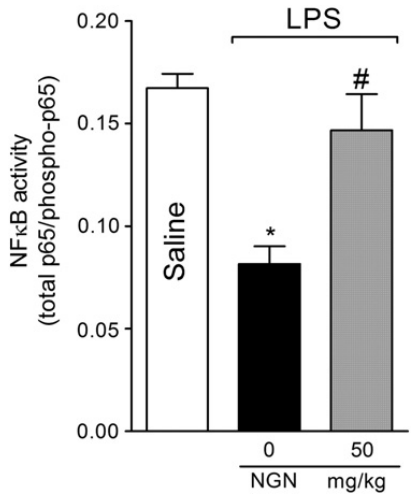

Fig. 8. Naringenin (NGN) inhibits NF-κB activation in paw skin induced by LPS injection. Samples from the paw skin tissue were collected 3 h after injection, and the activation of NF-κB was determined by ELISA. Results are means ± SEM, n = 6 mice per group per experiment, two independent experiments [*P < .05 vs. saline control; #P < .05 vs. 0 mg/kg (vehicle), (ANOVA followed by Tukey's test)].

4. Discussion

Pain is one of the most prominent complaints of patients with infectious diseases. It has been described that even small doses of LPS, which is found in large quantities in Gram-negative bacteria, increase pain sensation in humans [17]. Our results suggest a role for neutrophil and

macrophage recruitment and activation in hyperalgesia induced by LPS, and that naringenin (NGN) reduces inflammatory pain through inhibition of these inflammatory events. To kill bacteria, activated neutrophils degranulate and release bacteriolysis-inducing mediators such as myeloperoxidase (MPO) [15]. In a sense, this inflammatory response can be harmful as the induction of bacteriolysis also promotes the release of large amounts of bacterial cell wall components. LPS represents 70% of gram-negative bacterial cell wall composition, and the release of LPS after bacteriolysis could be a detrimental response to the host due to its pro-inflammatory activity. In fact, the bacteriolysis induced by neutrophil-derived mediators or by beta-lactam antibiotics, despite of killing bacteria also contributes to sustain inflammation and to post-infectious sequelae [37] and [38]. Thus, these results suggest that NGN exhibits a clinically relevant activity that deserves to be investigated in detail. In the present study, the overall quantity of LPS is not increased due to the action of recruited cells because we do not use living bacteria, permiting us to better describe the effects of host-related mediators. During inflammation, neutrophils and macrophages produce mediators capable of activating and sensitizing nociceptive neurons, including reactive oxygen species and cytokines [31], [32], [33], [34], [35] and [36]. Considering the key role of these mediators in producing inflammatory pain in several animal models, we aimed to evaluate whether NGN treatment could modulate oxidative stress and cytokine production as mechanisms of blocking hyperalgesia.

It has been described that NGN is an antioxidant molecule [4] which may be responsible for its protective effects observed here in reducing oxidative stress induced by LPS. However, the inhibitory effects of NGN on neutrophil and macrophage recruitment to the paw skin observed here also support the contribution of an anti-inflammatory activity resulting in inhibition of oxidative stress in this model. NGN also reduced TNF-α, IL-1β, IL-6, and IL-12 production in the mice paw, indicating that inhibiting cytokine production is an important additional mechanism of this flavonoid. These cytokines are produced by neutrophils and macrophages, and play important roles in driving inflammatory pain [35], [39] and [40]. Since previous data indicated the relationship between cytokines and oxidative stress systems in the induction of inflammatory hyperalgesia [41]. NGN mediated control of both may contribute to the inhibition of inflammatory hyperalgesia.

Macrophages are tissue-resident cells of the innate immune system. These cells respond rapidly to LPS by releasing pro-inflammatory mediators that activate endothelial cells and

promote the recruitment of neutrophils to the site of injury in a NF-κB-dependent manner [42], [43] and [44]. Moreover, macrophage-derived products, such as IL-1β and TNF-α, together with LPS, extend the survival of recruited neutrophils and, thus, contribute to tissue damage and hyperalgesia [45]. Taking into account the above described key role of macrophages during the initial steps that lead to an inflammatory response, the effects of naringenin was evaluated during LPS-induced macrophage activation. We observed that NGN treatment inhibited NF-κB activation in vitro in RAW 264.7 macrophages without inducing cytotoxicity or compromising cell viability as well as in vivo in the paw skin of mice. Taken together, these results suggest that NGN targets NF-κB to inhibit inflammatory pain.

It was demonstrated that TLR4 activation by intraplantar injection with LPS in mice induces acute hyperalgesia in a MyD88-dependent and TRIF-independent manner [18]. We demonstrated that NGN inhibits LPS-induced NF-κB activity which correlates with reduced levels of hyperalgesic mediators (TNF-α, IL-1β, IL-6, IL-12, and superoxide anion). Thus, it is plausible that NGN inhibits LPS-induced hyperalgesia and inflammatory cell recruitment by inhibiting the production of these mediators by macrophages. Activated neutrophils also release superoxide anion and hyperalgesic cytokines in large amounts in a NF-κB-dependent manner, and inhibiting the recruitment of these cells by NGN treatment is also a mechanism that certainly contributes to reducing inflammatory pain. It was demonstrated recently that LPS activates the transient receptor potential cation channel TRPA1 directly on nociceptive neurons to induce neurogenic inflammation [46]. NGN can enhance the activity of TRPA1 in enteroendocrine cells [47], however, it remains to be determined whether NGN affects nociceptive neuron activity through TRPA1 modulation. The ability of NGN to inhibit LPS-induced inflammatory pain and leukocyte recruitment was related to inhibition of macrophages by targeting superoxide anion release, cytokine production (TNF-α, IL-β, IL-6, and IL-12), and NF-κB activation. Moreover, NGN partially inhibited the recruitment of leukocytes to the peritoneal cavity, which represents an important effect of reducing inflammation without abolishing the immune response during infectious diseases.

In conclusion, the present study has demonstrated the analgesic and anti-inflammatory potential of the flavonoid NGN both in vivo and in vitro. The antinociceptive action of NGN observed here in models of mechanical and thermal hyperalgesia may depend on inhibition of leukocyte recruitment and activation (macrophages and neutrophils), oxidative stress (increased antioxidant capacity), and cytokine production (TNF-α, IL-1β, IL-6 and IL-12).

Furthermore, the anti-inflammatory effect of NGN was demonstrated in peritoneal exudate (inhibition of recruitment and activation of leukocytes, superoxide anion production and lipid peroxidation and TNF-α, IL-1β, IL-6 and IL-12 production) and in RAW 264.7 macrophage cell lineage cultures (inhibition of NFκB activation, superoxide anion, TNF-α, IL-1β, IL-6 and IL-12 production without cytotoxicity association in tested concentrations). The observed phenotype was related to NGN inhibition of NF-κB activation. These data indicate that NGN may be a promising compound to treat inflammatory pain in patients with inflammatory diseases, and consequently, merits further preclinical and clinical investigations.

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4 CONCLUSÕES GERAIS

O tratamento com naringenina reduz a dor inflamatória induzida por diversos estímulos através da inibição da via inflamatória do NF-κB e da ativação da via analgésica do óxido nítrico (Figura 5). O uso prolongado da naringenina não provocou efeitos adversos de toxicidade gástrica e hepática, enfatizando o potencial terapêutico da naringenina no tratamento da dor inflamatória.

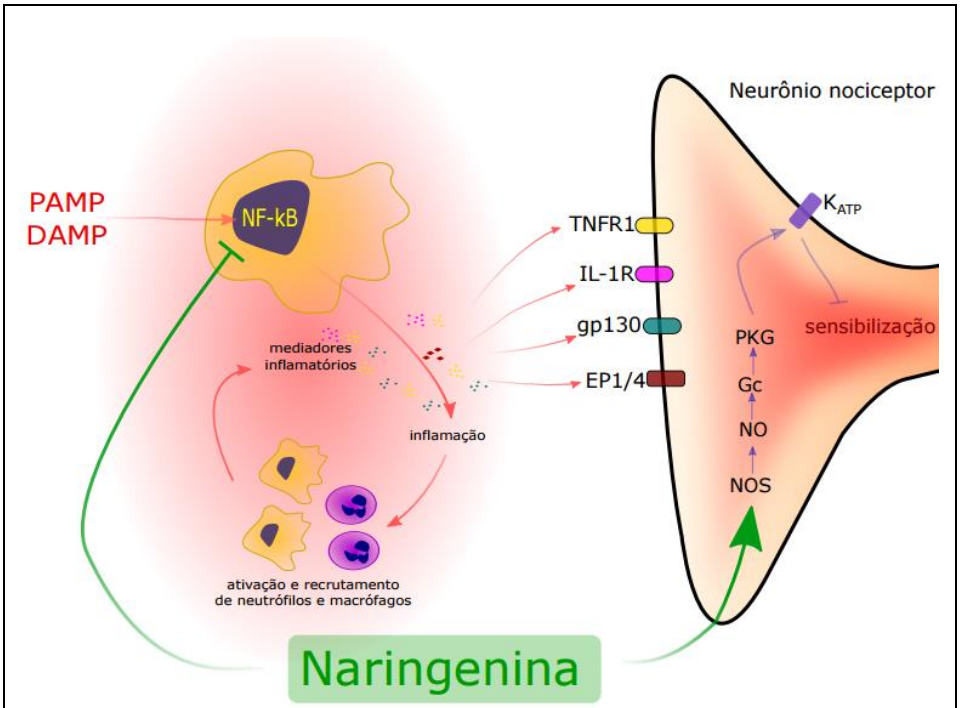

Figura 5. Mecanismos anti-inflamatórios e analgésicos da naringenina.

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