Quercetin inhibits gout arthritis in mice: induction of an opioid-dependent regulation of inflammasome

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Abstract We investigated the anti-inflammatory and analgesic effects of quercetin in monosodium urate crystals (MSU)-induced gout arthritis, and the sensitivity of quercetin effects to naloxone, an opioid receptor antagonist. Mice were treated with quercetin, and mechanical hyperalgesia was assessed at 1–24 h after MSU injection. In vivo, leukocyte recruitment, cytokine levels, oxidative stress, NFκB activation, and gp91phox and inflammasome components (NLRP3, ASC, Pro-caspase-1, and Pro-IL-1β) mRNA expression by qPCR were determined in the knee joints at 24 h after MSU injection. Inflammasome activation was determined, in vitro, in lipopolysaccharide-primed macrophages challenged with MSU. Quercetin inhibited MSU-induced mechanical hyperalgesia, leukocyte recruitment, TNFα and IL-1β production, superoxide anion production, inflammasome activation, decrease of antioxidants levels, NFκB activation, and inflammasome components mRNA expression. Naloxone pre-treatment prevented all the inhibitory effects of quercetin over MSU-induced gout arthritis. These results demonstrate that quercetin exerts analgesic and anti-inflammatory effect in the MSU-induced arthritis in a naloxone-sensitive manner.

Keywords Monosodium urate crystals · Gouty arthritis · Quercetin · NLRP3 · Inflammasome · Cytokines · Inflammatory pain

Introduction

Gout is an arthropathy characterized by intense inflammation and pain induced by monosodium urate (MSU) crystal deposition into the articular joint and surrounding tissues (Khanna et al. 2012; McGettrick and O’Neill 2013). The interaction of MSU crystals with phagocytes such as macrophages and infiltrating leukocytes induces the secretion of various inflammatory mediators, consequently triggering a typical inflammatory response and increased pain sensitivity, provoking hyperalgesia (Verri et al. 2006; Martin et al. 2009; Zarpelon et al. 2013). It is well known that MSU crystals undergo phagocytosis followed by rupture of the phagolysosome and activation of NLRP3 inflammasome, which culminates in the release of the active form of IL-1β. It has also been reported that patients with gout arthritis show a significant response to IL-1.
inhibition, indicating the crucial role of IL-1β in driving intense inflammatory pathology (Lu et al. 2014). Furthermore, IL-1β activates synoviocyte-like macrophages promoting the release of additional pro-inflammatory cytokines such IL-8, IL-17, and TNFα (Martinon et al. 2006; Lu et al. 2014). Inflammatory cytokines such as IL-1β and TNFα activate the transcription factor NF-κB resulting in production of chemotactic molecules including IL-8 to induce neutrophil chemotraction (Martinon et al. 2006; Neuschäfer-Rube et al. 2013; Dhanasekar et al. 2015).

The current therapeutic approaches against gouty arthritis are given mainly by the treatment of inflammation and the control of hyperuricemia. For instance, allopurinol acts by inhibiting hepatic xanthine oxidase enzyme (XOD) activity and then reducing serum uric acid levels. However, allopurinol is not able to control the acute gout attacks (Dubchak and Falasca 2010), and also can induce several side effects such as fever, skin rashes, allergic reactions, hepatitis, and nephropathy (Umamaheswari et al. 2009).

Thus, non-steroidal anti-inflammatory (NSAIDs) drugs (e.g., indomethacin) and alkaloid drugs (e.g., colchicine) are frequently used as the first-line approach therapies for treatment of acute gout inflammation. Nevertheless, these classes of drugs induce severe side effects such as gastrointestinal and renal toxicity, and gastrointestinal bleeding (Cronstein and Terkeltaub 2006). Immunobiological therapies targeting IL-1β are also useful in the treatment of gout arthritis attack, but the high cost reduces its wide clinical use (Dubchak and Falasca 2010). Thus, the research on novel drugs to treat gout arthritis has been a growing focus of interest (Sabina et al. 2011; de Souza et al. 2012; Rukdee et al. 2015; Zhao and Huang 2015; Xu et al. 2016). Therapeutic natural compounds are a growing field, since current therapies have relayed on natural compounds to develop efficacious treatments such as morphine, capsaicin, salicylate, and menthol (Julius 2013).

Flavonoids are multi-target molecules that inhibit inflammation and oxidative stress (Pinho-Ribeiro et al. 2015; Martinez et al. 2015a, b; Sun et al. 2016; Kim and Park 2016; Manchope et al. 2016; Liu et al. 2016). Evidence supports the safety of flavonoids due to low or no toxic effect contrasting to non-steroidal anti-inflammatory drugs (Okamoto 2005; Pinho-Ribeiro et al. 2016). Quercetin, a bioflavonoid presenting low toxicity (Okamoto 2005) and several biological properties such as antioxidant and anti-inflammatory (Valério et al. 2009; Wang et al. 2012; Guazelli et al. 2013; Sun et al. 2015a), has been a focus of varied studies on pain and inflammation (Anjaneyulu and Chopra 2003a; Valério et al. 2009; Souto et al. 2011; Calixto-Campos et al. 2015). Quercetin acts in an opioid-dependent manner inducing analgesia in diabetic- and cancer-induced pain (Anjaneyulu and Chopra 2003a; Calixto-Campos et al. 2015). Quercetin inhibits MSU-induced knee joint edema, histological leukocyte infiltration, IL-1β, TNFα, prostaglandin E2 and nitric oxide production, cyclooxygenase-2 expression, and lipid peroxidation. Furthermore, quercetin treatment reverted MSU-induced reduction of superoxide dismutase, catalase, and glutathione peroxidase activities in rats (Huang et al. 2012). However, it remains to be determined the molecular mechanisms of gout arthritis physiopathology that are targeted by quercetin.

This study aimed to investigate the effect of quercetin treatment in experimental gout arthritis induced by intra-articular injection of MSU assessing its analgesic, anti-inflammatory and antioxidant effects, including the NFκB and NLRP3 inflammasome pathways. Hence, we evaluated the contribution of endogenous opioid in the effect of quercetin.

Materials and methods

Animals

The experiments were performed with male Swiss mice (20–25 g, Universidade Estadual de Londrina, Londrina, PR, Brazil) housed in standard clear plastic cages (5–6 per cage) with free access to food and water. All testing was performed between 9:00 a.m. and 5:00 p.m. in a temperature-controlled room. Animal care and handling procedures were performed with the approval of the Ethics Committee of the Universidade Estadual de Londrina (Process Number 13279.2011.76) and followed the International Association for the Study of Pain guidelines as described by Zimmermann (1983). All efforts were made to minimize the number of animals used and their suffering.

Drugs and reagents

The following materials were obtained from the sources indicated: Quercetin at 95% purity from Acros (Pittsburg, PA); MSU and naloxone were from Sigma-Aldrich (St Louis, MO); Boric acid was from Promega, and uricase/fasturtec was from Sanofi-Synthelabo. ELISA kits to determine murine TNF-α and IL-1β were obtained from eBioscience (San Diego, CA).

MSU crystal preparation

MSU crystals were prepared according to the method described previously (Nishimura et al. 1997). In brief, 800 mg of monosodium urate was dissolved in 155 mL of boiling water containing 5 mL of 1 N NaOH. After the pH was adjusted to 7.2, the solution was gradually cooled by
stirring at room temperature. The crystals were collected by centrifugation at 3000 g for 2 min at 4 °C. The crystals were evaporated and sterilized by heating at 180 °C for 2 h and stored in a sterile environment until use.

Experimental protocols

In the first set of experiments, mice were treated with quercetin (10–100 mg/kg, s.c., 30 min) or vehicle (20% tween saline) before the MSU injection (100 µg/10 µL per knee joint) to determine the dose–response of quercetin (Fig. 1a). The intensity of mechanical hyperalgesia was measured 1–24 h after MSU injection by the electronic pressure-meter test. To determine the participation of endogenous opioid on quercetin analgesic and anti-inflammatory effects, mice were pretreated with naloxone (0.3–3 mg/kg, i.p., 1 h) before quercetin treatment (100 mg/kg, s.c.) or vehicle. The inflammatory parameters were evaluated at 24 h after injection of the MSU crystals. After 24 h, the articular cavities were washed with saline/EDTA solution for leukocytes counts and differential determination. Periarticular tissues were removed from the joints for evaluation of synovitis in articular tissue, cytokines, NfκB activation, inflammasome components mRNA expression (NLRP3, ASC, Pro-caspase-1 and Pro-IL-1β), evaluation of antioxidant capacity (GSH, FRAP, and ABTS assay), superoxide anion production (NBT reduction), Gp91phox, Nrf2 and HO-1 by qPCR. In vitro (Fig. 1b), bone marrow-derived macrophages (BMDMs) were cultured in 96-well plates. The activation of BMDMs first signal was induced by LPS (500 ng/mL)–primed BMDMs was incubated during 3 h, after that naloxone (0.2–30 µM) incubated with BDMNs cells 1 h before quercetin treatment, in addition, quercetin (0.5–30 µM) or morphine (0.2–20 µM) treatment performed during 30 min before stimulation with MSU crystals (450 µg/mL) and incubated during 5 h. IL-1β levels in the culture supernatant were measured.
Electronic pressure-meter test (mechanical hyperalgesia)

Evaluation of mechanical hyperalgesia was performed as described previously (Guerrero et al. 2006) using an electronic pressure-meter (INSIGHT Instruments). The flexion withdrawal threshold was used to in behavioral responses associated with pain. Results are expressed in grams force (g).

Leukocyte total and differential counts

To determine the leukocyte recruitment to the articular cavities, joint cavities were washed three times with 3.3 μL of PBS containing 1 mM EDTA. The total numbers of leukocytes were determined in Neubauer chamber diluted in Turk’s solution. Differential cell counts were determined in cytocentrifuge Rosenfeld stained slices (Cytospin 4; Shandon, Pittsburg, PA). Total and differential cell counts were performed with a light microscope and the results were expressed as the number (mean ± SEM) of total of leukocytes or neutrophils \( \times 10^3 \) per cavity (Verri et al. 2010).

Histopathological analysis

Mice were sacrificed 24 h after MSU injection. The articular joint of mice was removed, fixed with 10% paraformaldehyde in PBS, and then decalcified for 10 days with EDTA and embedded in paraffin for histological analysis. The paraffin sections were stained with hematoxylin and eosin for the conventional morphological evaluation. Dimension used for the analysis was 616 \( \times \) 662 pixels for analysis (Field) and magnification 400x and the arrow indicate representative infiltrate inflammatory cells counted.

Cytokine production

At 24 h after the i.a. injection of MSU, animals were terminally anaesthetized, and the samples of knee joints were removed and homogenized in 500 μL of Trizol reagent and centrifuged (12,000 rcf \( \times \) 15 min \( \times \) 4 °C), and total RNA was measured with a spectrophotometer and the wavelength absorption ratio (260/280 nm) was between 1.8 and 2.0 for all preparations and extracted using the SV Total RNA Isolation System (Promega) (Verri et al. 2008). All reactions were performed in triplicate using the following cycling conditions: 50 °C for 2 min, 95 °C for 2 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 30 s. qPCR was performed in a LightCycler Nano Instrument (Roche, Mississauga, ON, USA) sequence detection system using the Platinum SYBR Green qPCR SuperMix UDG (Invitrogen, USA). The mRNA level of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control. The primers used were Gapdh forward: CAT ACC AGG AAA TGA GCT TG, reverse: ATG ACA TCA AGA AGG TGG TG; Nrf2, forward: TCA CAC GAG ATG AGC TTA GGG CAA, reverse: TAC AGT TCT GGG CGG CGA CTT TAT; Gp91phox, forward: AGC TAT GAG GTG ATG TTA GTG G, reverse: CAC ATT ACC AGA CAG ACT TGA G; Nr1f3, forward: AGC TAT GAG GTG ATG TTA GTG G, reverse: CAC CAA TTA GTC AGA AGA CAG ACT TGA G; HO-1, forward: CCC AAA ACT GGC CTT TAA AA, reverse: CGT GTG CAG TCA ACA TGG AT; Pro-caspase-1: forward: TGG TCT TGT GAC TTG GAG GA, reverse: TGG CTT CTT ATT GGC AC G AG AT; Pro-IL-1β, forward: GAA ATG CCA CCT TTT GAC AGT G, reverse: TGG ATG CTC TCA TCA GGA CAG; ASC, forward: ATG GGG CGG GCA CGA GAT G, reverse: GCT CTG CTC CAG GTC CAT CAC. The SYBR green PCR Master Mix was used according to the manufacturer’s instructions.

Reduced glutathione (GSH) measurement

At 24 h after the i.a. injection of MSU, animals were terminally anaesthetized and the samples of knee joints were removed and maintained at −80 °C for at least 48 h. The samples were homogenized with 200 μL of 0.02 M EDTA. The homogenate was mixed with 25 μL of 50% trichloroacetic acid and was homogenized three times during homogenates were centrifuged (14,000 rpm \( \times \) 10 min \( \times \) 4 °C), with the supernatants used to assess the levels of phosphorylated and total NF-kB p65 subunit by ELISA using PathScan® kits (Cell Signaling) according to the manufacturer’s directions. The results represent the sample ratio (total p65/phospho-p65) measured at 450 nm (Multiskan GO Thermo Scientific).
15 min. The mixture was centrifuged (15 min × 1500g × 4 °C). The supernatant was added to 200 µL of 0.2 M TRIS buffer, pH 8.2, and 10 µL of 0.01 M DTNB. After 5 min, the absorbance was measured at 412 nm against a reagent blank with no supernatant. A standard curve was performed with standard GSH. The results are expressed as GSH per milligram of tissue (Sedlak and Lindsay 1968; Borghi et al. 2013; Staurengo-Ferrari et al. 2014).

Nitrobluetetrazolium (NBT) test

The quantitation of O$_2^-$ production in tissue homogenate (10 mg/mL in 1.15% KCl) was performed using the nitroblue tetrazolium test (NBT). Briefly, 50 µL of homogenate tissue was incubated with 100 µL of NBT (1 mg/mL) in 96-well plates at 37 °C for 1 h. The supernatant was then removed and the reduced formazan solubilized by adding 120 µL of 2 M KOH and 120 µL of DMSO. The NBT reduction was measured using a Multiscan spectrophotometer at 600 nm. The results were expressed as optical density per milligram of tissue.

Ferric-reducing ability potential (FRAP) and free-radical scavenging ability (ABTS) assays

The samples of knee joints were collected, immediately homogenized with 500 µL of 1.15% KCl, and centrifuged (10 min × 200g × 4 °C). The ability of the sample to resist oxidative damage was determined as ferric-reducing ability using the FRAP assay and as free-radical scavenging ability using the ABTS assay (Borghi et al. 2013). For the FRAP assay, 50 µL of supernatant was mixed with 150 µL of deionized water and 1.5 mL of freshly prepared FRAP reagent. The reaction mixture was incubated at 37 °C for 30 min, and the absorbance was measured at 595 nm. For the ABTS assay, the ABTS solution was diluted with phosphate buffer saline at pH 7.4 to an absorbance of 0.80 at 730 nm. Then, 1.0 mL of diluted ABTS solution was mixed with 20 µL of supernatant. After 6 min, the absorbance was measured at 730 nm. The results were equated against a Trolox standard curve (1.5–30 µmol/L, final concentrations). The results are expressed as Trolox equivalents per milligram of tissue weight in both assays.

Preparation of bone marrow-derived macrophages (BMDMs) and inflammasome activation assay

Femora and tibiae of mice C57BL/6 mice (8 weeks old) were aspirated with RPMI1640 media. Bone marrow cells were cultured in RPMI 1640 medium containing 10% FBS and 15% L929 cell conditioned medium. BMDM were harvested at day 7 and plated at the density of 1.5 × 10^5 cells/well in 96-well plate. BMDM were stimulated with 500 ng/mL E. coli LPS (Santa Cruz Biotechnology) and 3 h later treated with 450 µg/mL of MSU to stimulate NLRP3 inflammasome activation as described previously (Martinon et al. 2006). BMDM were treated with quercetin (0.3; 3 or 30 µM), or morphine (0.2; 2 or 20 µM), 30 min before MSU stimulation. Naloxone (0.2; 2 or 20 µM) was added 1 h prior to quercetin treatment (Khabbazi et al. 2015). Supernatants were collected 5 h after MSU stimulation and IL-1β concentration quantitated by ELISA.

Data analyses

Data were analyzed using GraphPad Prism statistical software (GraphPad Software, Inc., USA-500.288, version 5.0). Results are presented as mean ± SEM of measurements made on six mice per group per experiment and are representative of two independent experiments. 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 < 0.05 was considered significant.

Results

Quercetin inhibits MSU-induced mechanical hyperalgesia and leukocyte recruitment in gout arthritis

Quercetin decreased the parameters of mechanical hyperalgesia and leukocyte recruitment; however, the dose of 10 mg/kg of quercetin affect MSU-induced mechanical hyperalgesia only at 1 h after MSU injection. On the other hand, the dose of 30 mg/kg inhibited at 1, 5, 7, and 24 h, and the dose of 100 mg/kg inhibited all points evaluated of mechanical hyperalgesia (Fig. 2a). Only dose of 100 mg/kg of quercetin inhibited the MSU-induced recruitment of total of leukocytes and neutrophils to the knee joint (Fig. 2b, c). Based on the results of Fig. 2, the dose of 100 mg/kg of quercetin was selected for next experiments (Fig. 2a, b).

Quercetin inhibits MSU-induced mechanical hyperalgesia and leukocyte recruitment in a naloxone-sensitive manner

The dose of 0.3 mg/kg of naloxone was ineffective in inhibiting the antinociceptive effect of quercetin in mechanical hyperalgesia test. On the other hand, the doses
of 1 and 3 mg/kg of naloxone prevented since the 1st h the antinociceptive effect of quercetin (Fig. 3a) in MSU-induced mechanical hyperalgesia. The pre-treatment with naloxone also reversed the quercetin inhibition of MSU-induced recruitment of total leukocytes (Fig. 3b) and neutrophils (Fig. 3c) only at the dose of 3 mg/kg. Thus, the quercetin inhibition of MSU-induced mechanical hyperalgesia and leukocyte recruitment depends on activation of opioid receptors.

**Quercetin reduces the synovitis in articular joint-induced by gouty arthritis in a naloxone-sensitive manner**

Quercetin inhibits MSU-induced synovitis in articular joint, decreasing the inflammatory infiltrate cells (Fig. 4a–f). In addition, quercetin effect was reversed by pre-treatment with naloxone at dose 3 mg/kg in a sensitive manner (Fig. 4f). The treatment with naloxone did not present effect per se.

**Quercetin inhibits MSU-induced TNF-α and IL-1β production in a naloxone-sensitive manner**

Quercetin inhibited MSU-induced production of TNF-α (Fig. 5a) and IL-1β (Fig. 5b). In turn, naloxone pre-treatment prevented quercetin inhibition of MSU-induced TNF-α and IL-1β production in the knee joints at 24 h (Fig. 5a, b). The treatment with naloxone did not present effect per se.

**Quercetin inhibits MSU-induced NFκB activation in a naloxone-sensitive manner**

Quercetin inhibited MSU-induced NFκB activation as observed by an increase of total NFκB/phosphoNFκB ratio. In turn, naloxone treatment prevented quercetin inhibition of MSU-induced NFκB activation (Fig. 6). The treatment with naloxone did not present effect per se.
Quercetin inhibits MSU-induced mRNA expression in a naloxone-sensitive manner

Quercetin inhibited MSU-induced mRNA expression of the inflammasome components NLRP3, ASC, pro-caspase-1, and pro-IL-1β (Fig. 7a–d) in the knee joints at 24 h post-stimulus injection as determined by qPCR. On the other hand, naloxone treatment prevented quercetin inhibition of MSU-induced mRNA expression of inflammasome components.

Quercetin inhibits MSU-induced antioxidant capacity, superoxide anion production, and gp91phox mRNA expression in a naloxone-sensitive manner

Quercetin inhibited MSU-induced reduction of antioxidant defenses as observed by maintenance of GSH levels (Fig. 8a), ferric-reducing ability potential (FRAP assay—Fig. 8b), and free-radical scavenging ability (ABTS assay—Fig. 8c). Quercetin also inhibited MSU-induced NBT reduction (superoxide production—Fig. 8d) assessment and gp91phox mRNA expression (Fig. 8e) in the knee joints. In turn, quercetin effects were inhibited by naloxone treatment (Fig. 8a–e). The treatment with naloxone did not present effect per se.

Quercetin induces Nrf2 and HO-1 mRNA expression in MSU-induced arthritis in a naloxone-sensitive manner

MSU stimulus did not alter Nrf2 (Fig. 9a) or HO-1 (Fig. 9b) mRNA expression in the knee joints. On the other hand, quercetin induced significant increase of Nrf2 (Fig. 9a) or HO-1 (Fig. 9b) mRNA expression, which was inhibited by naloxone treatment.

Quercetin inhibits MSU-induced IL-1β levels in BMDMs supernatant in gouty arthritis in a naloxone-sensitive manner in vitro

LPS priming (first signal) followed by MSU stimulation (second signal) induced an increase of IL-1β levels in BMDM, which was inhibited by quercetin in a dose-dependent manner (Fig. 10a). Quercetin at the concentration...
of 30 μM prevented inflammasome activation by MSU crystals decreasing IL-1β levels in BMDMs supernatant. In a similar way, morphine treatment also inhibited NLRP3 activating when stimulated with MSU crystals at the concentration of 20 μM (Fig. 10c). In turn, NLPR3 inflammasome stimulation in the presence of naloxone at concentration (20 μM) reversed the quercetin effect (Fig. 10b).

 Discussion

Monosodium urate (MSU) crystals activate phagocytes in the joints leading to the development of inflammatory response due to activation of NF-κB and NLRP3 inflammasome. These signaling pathways promote, for instance, the production of IL-1β resulting in the influx of neutrophils into the affected articular space. In fact, neutrophil recruitment is a major feature of acute gout arthritis provoking synovial membrane damage and release of lysosomal enzymes, elastase, oxygen-derived free radicals, chemotactic factors, and pro-inflammatory cytokines, which lead to enhanced inflammatory response and pain (Desaulniers et al. 2001; Cronstein and Terkeltaub 2006; Huang et al. 2012). The present data show that MSU-induced gout arthritis promotes mechanical hyperalgesia, leukocyte recruitment, TNF-α and IL-1β cytokine production, as well as NFκB activation, up regulation of NLRP3, ASC, Pro-caspase-1, and pro-IL-1β mRNA expression, and oxidative stress. All these MSU-induced physiopathological alterations in the joints were inhibited by the flavonoid quercetin in a naloxone-sensitive manner.

Quercetin presents analgesic effect, decreasing the pain threshold and leukocyte recruitment in varied pain models.

Fig. 4 Quercetin inhibits inflammatory infiltrate cells on synovial tissue in an opioid-dependent manner. Treatment with naloxone (3 mg/kg, i.p.) was performed 1 h before quercetin (100 mg/kg, s.c.) or vehicle (Tween 80 plus saline), and after additional 30 min, mice received MSU injection (100 μg/10 μL, i.a.). The joint samples were collected at 24 h after MSU injection. Control (a), MSU (b), MSU and quercetin (c), MSU and quercetin and naloxone (d), and MSU and naloxone (e) and inflammatory infiltrate cells analysis/fields (f). All sections were stained with Hematoxylin and Eosin (magnification a-f, ×400) and the figure is representative of all experiment. Dimension used was 616 × 662 pixels for analysis (Field). Scale bars 50 μm. The arrow indicates representative infiltrate inflammatory cells counted. Results are presented as mean ± SEM of six mice per group per experiment, and are representative of two separated experiments. *P < 0.05 compared to saline group; **P < 0.05 compared to MSU group and ***P < 0.05 compared with Quercetin group. ANOVA followed by Tukey’s t test.
such as Ehrlich tumor-induced cancer pain (Calixto-Campos et al. 2015), streptozotocin-induced diabetic neuropathic pain (Anjaneyulu and Chopra 2003a), superoxide anion-induced pain (Maioli et al. 2015), lipopolysaccharide (LPS)-induced hyperalgesia (Anjaneyulu and Chopra 2003b), and carrageenan-induced hyperalgesia (Vale´rio et al. 2009) and leukocyte recruitment (Souto et al. 2011). Therefore, the analgesic effect of quercetin is consistent among varied models. However, it was not known, to our knowledge, whether quercetin would have analgesic effect in the gout arthritis model. Quercetin treatment inhibited MSU-induced mechanical hyperalgesia at all time-points concomitantly with a reduction of leukocyte recruitment in a dose-dependent manner. Despite the description that recruited neutrophils contribute to inflammatory hyperalgesia (Cunha et al. 2008), the inhibition of hyperalgesia and neutrophil recruitment lined up with the dose of quercetin of 100 mg/kg, while the dose of 30 mg/kg inhibited only the MSU-induced hyperalgesia, which suggests that the inhibition of neutrophil recruitment and hyperalgesia are not fully interdependent. There is evidence that quercetin inhibits MSU-induced edema and leukocyte recruitment by inhibiting cytokine production and oxidative stress (Huang et al. 2012).

The previous studies identified that the analgesic effect of quercetin depends on endogenous opioids and, therefore, is reversed by the opioid receptor antagonist naloxone.
However, it is unknown if this is the mechanism by which quercetin inhibits MSU-induced inflammation. In the present study, naloxone reversed, in a dose-dependent manner, the quercetin reduction of MSU-induced mechanical hyperalgesia and leukocyte recruitment. Opioids can act by three different mechanisms. First, opioids inhibit neuronal adenilate cyclase resulting in the reduction of PGE2-induced activation of adenilate cyclase and hyperalgesia; second, opioids act on peripheral nociceptor neurons activating the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT)/neuronal nitric oxide synthase (nNOS)/nitric oxide (NO)/cyclic guanosine monophosphate-dependent protein kinase (PKG)/ATP-sensitive potassium channel (KATP) signaling inducing hyperpolarization of nociceptors (Cunha et al. 2010); and third, opioids inhibit cytokine production and the consequent hyperalgesia and neutrophil recruitment (Wang et al. 2005; Clark et al. 2007; Martin et al. 2010).

In experimental conditions related to opioid tolerance, opioids can enhance pain through increased TLR4 signaling and cytokine production (Ellis et al. 2016; Liang et al. 2016). On the other hand, in regular clinical conditions, opioids induce analgesia and inhibit inflammation. For instance, the μ-opioid receptor agonist U50,488H inhibited myocardial ischemia and reperfusion injury-induced TLR4 expression, NFκB activation, myeloperoxidase (MPO) activity, and TNF-α production (Cai et al. 2014). The μ opioid receptor agonist morphine reduces TLR4 expression by macrophages. In turn, the μ opioid receptor antagonist naltrexone increases macrophage TLR4 expression, which indicates endogenous role of μ opioid receptors in regulating TLR4 expression (Franchi et al. 2012). Thus, opioid receptor activation has been related to consistent anti-inflammatory effects.

The anti-inflammatory and analgesic effects of quercetin depend on inhibiting NFκB activation (Chen et al. 2005; Vicentini et al. 2011; Borghi et al. 2016), which
corroborates the quercetin inhibition of MSU-induced cytokine production in rats and mice (Huang et al. 2012).
Nevertheless, our data expand these findings demonstrating that quercetin inhibition of NFκB activation is naloxone-sensitive and is an underlying mechanism by which quercetin reduced MSU-induced inflammation and pain. As a result of NFκB inhibition, quercetin inhibited MSU-induced NLRP3, ASC, pro-caspase-1, and pro-IL-1β mRNA expression. 

In BMDM primed with LPS, the challenge with MSU induces IL-1β release in the supernatant, which was inhibited by quercetin in a naloxone-sensitive manner. The in vitro system used in the present study allows to determine whether after signal 1 activation with LPS, a test drug can inhibit signal 2 that results in IL-1β maturation and release in the supernatant of cell culture (Martinon et al. 2006). The present data show that quercetin inhibits both signal 1 and 2, which explains the major anti-inflammatory and analgesic effect of quercetin in MSU-induced arthritis. Quercetin also inhibited NLRP3 inflammasome components expression and decreased IL-1β production in models of hepatic inflammation (Zhang et al. 2015), carrageenan-induced paw inflammation (Valério et al. 2009), renal injury (Hu et al. 2012), and spinal cord injury (Zhou et al. 2010; Jiang et al. 2016), although these studies did not investigate whether quercetin would be able to inhibit signal 2 as performed in the present work. In agreement with the present data, it has recently been demonstrated that quercetin inhibits ATP, nigericin, alum (NLRP3 activators), and double strand DNA (AIM2 activator)-induced inflammasome activation by diminishing ASC oligomerization (Domiciano et al. 2017).

Reactive oxygen species (ROS) contribute to acute and chronic inflammation (Rasool and Varalakshmi 2006).
MSU-induced arthritis promoted oxidative stress observed as decrease of GSH levels, FRAP and ABTS activities, and increase of NBT reduction (superoxide anion production) and gp91 phox mRNA expression. Quercetin inhibited MSU-induced oxidative stress in a naloxone-sensitive manner. Quercetin has inherent antioxidant chemical groups that can explain its antioxidant effects in vitro and in vivo (Verri et al. 2012). This flavonoid inhibits ROS such as hydrogen peroxide (H₂O₂) (Boumaza et al. 2016) and decreases gp91 phox gene expression (Sun et al. 2015b; Borghi et al. 2016) in models of inflammation, and also attenuates lipid peroxidation and increases antioxidant enzymes in gout arthritis models in rats (Huang et al. 2012). Importantly, quercetin acting on oxidative stress sensitive signaling pathways induces Nrf2 and HO-1 gene expression (Yao et al. 2007; Liu et al. 2015) enhancing the expression of multiple antioxidant enzymes such as SOD and GSH, thus decreasing oxidative stress (Jung and Kwak 2010; Bryan et al. 2013), which contributes to the resolution of oxidative stress damages. The sensitivity to
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