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Quercetin Reduces Inflammatory Pain: Inhibition of Oxidative Stress and Cytokine Production

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Quercetin (**1**) is known to have both antioxidant and antinociceptive effects. However, the mechanism involved in its antinociceptive effect is not fully elucidated. Cytokines and reactive oxygen species have been implicated in the cascade of events resulting in inflammatory pain. Therefore, we evaluated the antinociceptive mechanism of **1** focusing on the role of cytokines and oxidative stress. Intraperitoneal and oral treatments with **1** dose-dependently inhibited inflammatory nociception induced by acetic acid and phenyl-*p*-benzoquinone and also the second phase of formalin- and carrageenin-induced mechanical hypernociception. Compound **1** also inhibited the hypernociception induced by cytokines (e.g., TNFα and CXCL1), but not by inflammatory mediators that directly sensitize the nociceptor such as PGE_2 and dopamine. On the other hand, **1** reduced carrageenin-induced IL-1β production as well as carrageenin-induced decrease of reduced glutathione (GSH) levels. These results suggest that **1** exerts its analgesic effect by inhibiting pro-nociceptive cytokine production and the oxidative imbalance mediation of inflammatory pain.

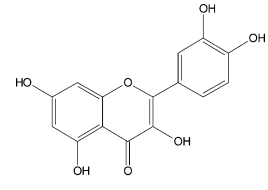
Quercetin (**1**) is the prototype antioxidant flavonoid because it exhibits all three structural components necessary for antioxidant activities of this compound class including (a) the presence of a catechol group in ring B, which has electron-donating properties and is a radical target; (b) a 2,3-double bond conjugated with the 4-oxo group, which is responsible for electron delocalization; and (c) the presence of both 3- and 5-hydroxy scavenging groups.[1] These structural characteristics of **1** explain why it has the highest antioxidant activity when compared to other flavonoids. In fact, the most widely recognized biological effects of **1** are related to the antioxidant properties that occur by scavenging oxygen radicals, inhibiting lipid peroxidation as well as protein and DNA oxidation, and chelating metal ions.[1,2]

Regarding the antinociceptive effect of **1**, it has been demonstrated that **1** inhibits acetic acid-induced writhing,[3] as well as formalin-induced overt pain-like behavior,[3] and attenuates thermal[4,5] and cold hypernociception in streptozotocin-induced diabetic neuropathy in rats.[5] These antinociceptive effects of **1** have been associated with activation of GABA and serotonin receptors, nitric oxide,[3] and endogenous opiates.[4] However, there are still some essential nociceptive mechanisms of **1** worthy of evaluation.

In terms of inflammatory pain, the sensitization of primary nociceptive neurons (nociceptors) occurs during inflammation, producing an increase in pain sensation (hypernociception). This sensitization is caused by direct action of inflammatory mediators such as prostaglandins (e.g., PGE_2, PGI_2) and sympathetic amines (e.g., dopamine, epinephrine) on their receptors present in the membrane of nociceptors. It is also accepted that the release of these direct-acting hypernociceptive mediators is preceded by the release of a cascade of cytokines.[6,7] In the carrageenin model of paw inflammation, this cascade is initiated with the release of TNFα and the chemokine CXCL1. These trigger the production of IL-1β, which in turn induces prostaglandin production. CXCL1 is also responsible for the stimulation of the sympathetic component of inflammatory pain.[8] There is also evidence that the role of cytokines in the production of final mediators, such as PGE_2, is preceded by the recruitment of neutrophils.[9,10]

Another important component of inflammatory pain concerns oxidative stress products, including superoxide anion, peroxynitrite, and hydrogen peroxide.[11−13] Usually, oxidative stress is kept under the control of the endogenous antioxidant system, which is constituted by different enzymes and substances such as superoxide dismutase, glutathione reductase, and reduced glutathione (GSH). However, during the inflammatory process, oxidative stress increases at a rate that overwhelms the capability of endogenous antioxidants to oppose it. This imbalance results in oxidative stress product-mediated injury and might also mediate inflammatory pain enhancement. For instance, peroxynitrite injection in the rat paw induces hypernociception.[14] On the other hand, prevention of the increase in the levels of oxidative stress products at the site of the inflammatory process or even in the spinal cord reduced hypernociception.[11−14] Although quercetin is best known for its antioxidant effects, there is no evidence that the reestablishment of oxidative balance is part of the analgesic activity of this compound.

In view of the information presented above, it was investigated as to whether the antinociceptive effect of **1** depends on counteracting cytokines and oxidative stress mediation of inflammatory pain.



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Results and Discussion

Quercetin (1) Inhibits Overt Pain-like Behaviors Induced by Different Stimuli. In the first series of experiments, the antinociceptive effect of **1** was evaluated in acetic acid-, phenyl-*p*-benzoquinone (PBQ)-, and formalin-induced pain-like behaviors. Mice were treated with **1** (3−100 mg/kg, ip) 30 min before ip

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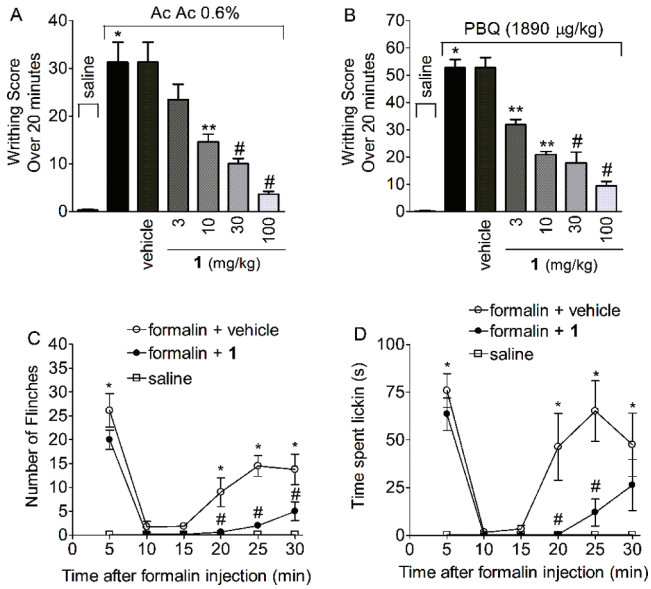

Figure 1. Quercetin (**1**) inhibits overt pain-like behaviors induced by different stimuli. Panels A and B: Mice were treated with **1** (3−100 mg/kg, ip, 30 min) or vehicle before an acetic acid (panel A) or phenyl-*p*-benzoquinone (PBQ, panel B) injection. The cumulative number of writhings (writhing score) was evaluated for 20 min ($n = 7$). Panels C and D: Mice were treated with **1** (100 mg/kg, ip, 30 min) or vehicle before the injection of formalin. The total number of flinches (panel C) and time spent licking the paw (panel D) were evaluated for 30 min and expressed in intervals of 5 min ($n = 6$) [$^{*}p < 0.05$ compared with the saline group, $^{**}p < 0.05$ compared to the vehicle group, and $^{\#}p < 0.05$ compared to the vehicle group and the dose of 3 mg/kg of **1** (one-way ANOVA followed by Tukey test)].

injection of acetic acid 0.6% (Figure 1A), phenyl-*p*-benzoquinone (1890 μg/kg, Figure 1B), or vehicle (saline or DMSO 2% diluted in saline, respectively). It was observed that **1** inhibited in a dose-dependent manner the acetic acid- and PBQ-induced writhings (Figure 1A and B). All doses of **1** inhibited the PBQ-induced writhing significantly, and the effects of the 30 and 100 mg/kg doses were significantly different compared to the 3 mg/kg dose (Figure 1A and B). In the formalin test (Figure 1C and D), treatment with **1** (100 mg/kg, 30 min before stimulus) did not alter the first phase of the response, but there was a significant reduction in the flinching and licking responses in the second phase at 20 and 25 min. At 30 min, there was a significant reduction in the number of flinches but not in the licking time (Figure 1C and D). These findings support a previous study that showed an antinociceptive effect of **1** in the acetic acid and formalin tests.[3] Herein, compound **1** inhibited the abdominal contortions induced by acetic acid or PBQ, which are dependent on the release of inflammatory mediators such as cytokines and prostanoids.[15,16] In agreement, **1** inhibited the second phase of the formalin test, which depends on cytokine production,[17] but not the first phase, which is considered to be a direct effect of formalin in TRPA1 receptors present in primary nociceptive neurons.[18] Therefore, it is possible that **1** could act by inhibiting prostanoid and/or cytokine production. The present result contrasts with a previous report that detected inhibition of both phases in the formalin test.[3] This difference is probably related to different doses, but the consistency of inhibition of the second phase may suggest that it represents the most relevant effect of **1**.

Treatment with Quercetin (1) via the Intraperitoneal and Oral Routes Reduced Carrageenin-Induced Mechanical Hypernociception. Next, the antinociceptive effect of **1** was tested in the carrageenin paw inflammation model. The ip treatment with **1** dose-dependently (3−100 mg/kg) inhibited carrageenin-induced mechanical hypernociception in the third hour up to 59% (Figure

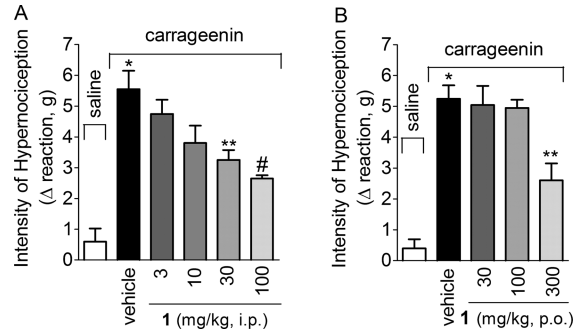

Figure 2. Treatment with quercetin (**1**) via ip and po routes reduced carrageenin-induced mechanical hypernociception. Mice were treated with **1** (3−300 mg/kg, 30 min) via ip (panel A) or po (panel B) routes or vehicle before the carrageenin injection. The intensity of hypernociception was measured 3 h after carrageenin injection by the electronic pressure-meter test ($n = 5$) [$^{*}p < 0.05$ compared with the saline group, $^{**}p < 0.05$ compared to the vehicle group and $^{\#}p < 0.05$ compared to the dose of 3 mg/kg of **1** (one-way ANOVA followed by Tukey test)].

2A). No significant effects were observed with 3 and 10 mg/kg doses of **1**, but significant reduction of carrageenin-induced hypernociception was observed with the 30 and 100 mg/kg doses. Additionally, the effect of 100 but not 30 mg/kg of **1** was significantly different than the dose at 3 mg/kg. It is known that the po absorption of **1** is very poor, and in fact, only the 300 mg/kg dose produced a significant antinociceptive effect on carrageenin (100 μg/paw)-induced mechanical hypernociception in the third hour by up to 55% (Figure 2B). The vehicle treatment (Tween 80 20% in saline) did not affect carrageenin hypernociception either po or ip (Figure 2A and B). Thus, **1** can also be used via the po route. Mice were also treated with **1** (100 mg/kg, ip route) at different time points (12, 6, 3, and 30 min) before carrageenin (100 μg/paw) injection. Only the 30 min pretreatment with **1** inhibited the mechanical hypernociception induced by carrageenin at 1, 3, and 5 h. The 3 h pretreatment with **1** inhibited the mechanical hypernociception induced by carrageenin at 3 h, whereas the other pretreatment time points were ineffective (Figure S1, Supporting Information). It is possible that the elapsed time of 12 or 6 h between the administration of quercetin and carrageenin injection allowed metabolism/excretion of **1**; consequently, there was not a sufficient amount of **1** to inhibit carrageenin-induced hypernociception. Nevertheless, even in a raw administration form, the effect of **1** would last approximately 6 h (30 min pretreatment plus 5 h effect or 3 h pretreatment plus 3 h effect). For the next group of experiments, a dose of 100 mg/kg of **1**, the ip route, and 30 min of pretreatment were used.

In addition to inflammatory nociception, **1** also inhibited carrageenin-induced paw edema at 0.5, 1, 2, and 3 h after stimulus injection (Figure S2, Supporting Information), supporting a wider potential therapeutic applicability of **1** in inflammatory conditions.

Quercetin (1) Reduced IL-1β Production without Altering Neutrophil Recruitment Induced by Carrageenin. Carrageenin-induced inflammatory hypernociception is mediated by a cascade of cytokines.[8] In turn, the hypernociceptive role of cytokines is mediated by the production of final sensitizing mediators such as PGE_2 and sympathetic amines.[8,18,19] This last step of the cascade seems to be dependent on the recruitment of neutrophils.[9,10] After mechanical hypernociception evaluation as shown in Figure 2A (third hour), mice were terminally anesthetized and the cutaneous plantar tissue was collected for myeloperoxidase (MPO) activity or cytokine measurement. There was no alteration in the MPO activity, disproving that **1** reduces hypernociception by inhibiting neutrophil recruitment (Figure S3A, Supporting Information). This absence of effect on leukocyte recruitment by **1** was unexpected since there is evidence that this flavonoid inhibits

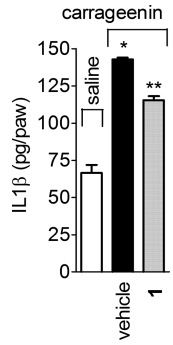

Figure 3. Quercetin (**1**) reduces carrageenin-induced IL-1β production. Mice were treated with **1** (100 mg/kg, ip, 30 min) or vehicle before the ipl injection of carrageenin. Three hours after carrageenin injection, mice were sacrificed and paw skin samples were collected for the determination of IL-1β production ($n = 5$) [$*p < 0.05$ compared with the saline group, and $**p < 0.05$ compared to the vehicle group (one-way ANOVA followed by Tukey test)].

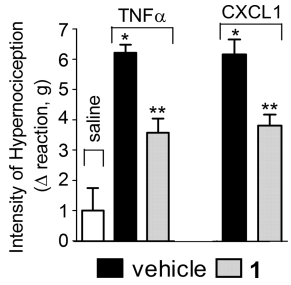

Figure 4. Quercetin (**1**) reduces TNFα- and CXCL1-induced mechanical hypernociception. Mice were treated with **1** (100 mg/kg, ip, 30 min) or vehicle before TNFα or CXCL1 injection. The intensity of hypernociception was measured 3 h after stimulus injection by the electronic pressure-meter test ($n = 5$) [$*p < 0.05$ compared with the saline group, $**p < 0.05$ compared to the vehicle group (one-way ANOVA followed by Tukey test)].

MPO activity in vitro[20] and in vivo.[21] On the other hand, **1** significantly inhibited the carrageenin-induced production of IL-1β (Figure 3) in the paw tissue by 36% but had no effect on TNFα or CXCL1 production (Figures S3B and S3C, Supporting Information, respectively). Thus, at least in part, the analgesic effect of **1** depends on the inhibition of IL-1β production.

Quercetin (1) Reduced TNFα- and CXCL1-induced Mechanical Hypernociception but Not Hypernociception Induced by Direct Acting Hypernociceptive Mediators. Mice were treated with **1** before TNFα (300 pg) or CXCL1 (20 ng) subcutaneous intraplantar (ipl) injection. Quercetin inhibited the mechanical hypernociception induced by TNFα (51%) and CXCL1 (43%) (Figure 4). Confirming the result obtained with carrageenin and MPO activity (Figure S3A, Supporting Information), **1** did not alter the increase of MPO activity induced by injection of TNFα and CXCL1 (Figure S4A, Supporting Information). Furthermore, treatment with **1** did not affect the mechanical hypernociception induced by the ipl injection of PGE_2 (100 ng/paw) or dopamine (10 μg/paw) (Figure S4B, Supporting Information), therefore giving no evidence that quercetin acts as an antagonist of PGE_2 and dopamine receptors or has a direct effect on primary nociceptive neurons. The inhibition of IL-1β production by **1** (Figure 3) corroborates the fact that quercetin also inhibited the mechanical hypernociception induced by TNFα and CXCL1, which depend on IL-1β production.[8] Thus, **1** inhibits a crucial step in the carrageenin-induced cytokine cascade. Further supporting IL-1β inhibition as the mechanism of action of quercetin, the hypernociception produced by the directly acting sensitizing mediators (PGE_2 and dopamine) was not altered by **1**. Therefore, it is

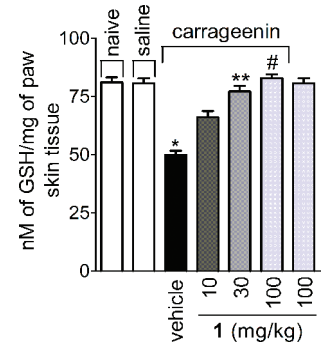

Figure 5. Quercetin (**1**) prevents the carrageenin-induced decrease of GSH levels. Mice were treated with **1** (10−100 mg/kg, ip, 30 min) or vehicle before the carrageenin or saline injection. The samples of subcutaneous plantar tissue were collected 3 h after stimulus and processed for reduced glutathione (GSH) levels measurement. Samples of 5 mice were pooled for processing and the measurements made in triplicate [$*p < 0.05$ compared with the saline group, and $**p < 0.05$ compared to the vehicle group (one-way ANOVA followed by Tukey test)].

suggested that quercetin is acting upstream of PGE_2 and dopamine sensitization of nociceptors and corroborates evidence that IL-1β induces hypernociception via PGE_2 production.[8] Although the mechanism by which **1** inhibits IL-1β production is not immediately apparent, there is evidence suggesting that this flavonoid could inhibit the activation of nuclear factor κB (NFκB), which is a crucial transcription factor involved in the production of IL-1β.[6] Furthermore, it cannot be ruled out that quercetin might act by additional mechanisms. For instance, there is evidence that **1** inhibits carrageenin-induced PGE_2 production in the air-pouch;[22] therefore, the inhibition of PGE_2 production can also play a significant role in the antinociceptive activity of this compound. Nevertheless, this could be an indirect effect as a result of the prevention of IL-1β production, which is an important inducer of COX-2 expression and, therefore, PGE_2 production.[23]

Quercetin (1) Prevented the Carrageenin-Induced Decrease in GSH Levels. In the last part of this study, it was evaluated as to whether or not the antinociceptive effect of **1** is associated with a reduction in oxidative stress observed during carrageenin inflammation. In fact, the hypernociceptive dose of carrageenin reduced the levels of GSH by 38%, which was reversed by treatment with **1** in a dose-dependent (10, 30, and 100 mg/kg) manner (Figure 5). Although the dose of 10 mg/kg of quercetin reduced GSH depletion, this effect was not significant. Concerning the dose of 30 mg/kg of **1**, the prevention of carrageenin-induced reduction of GSH levels was statistically significant, and the dose of 100 mg/kg abolished the carrageenin-induced depletion of GSH with significant differences compared with the 10 mg/kg dose. There was no significant modification of basal GSH levels by **1** alone, as seen in the last bar of Figure 5. Thus, besides the effect on IL-1β production, in the present study the results indicate that the antinociceptive activity of **1** is also associated with the reduction of oxidative stress. This hypothesis is based on the following evidence: (a) It was determined that the ipl injection of carrageenin at a hypernociceptive dose induces a decrease in GSH, indicating a role for oxidative stress in inflammatory hypernociception. In fact, GSH belongs to the endogenous antioxidant system that also includes enzymes such as superoxide dismutase and glutathione reductase and other substances such as ascorbic acid. The GSH directly scavenges free radicals by hydrogen transference and acts as a cofactor for the enzyme GSH-peroxidase, which in turn scavenges peroxides, finally regenerating vitamins E and C.[21] (b) Quercetin inhibited the carrageenin-induced mechanical hypernociception and reduction of GSH levels in a similar manner. (c) Free radicals produced as a result of oxidative stress are able to induce

hypernociception and have an endogenous role in inflammatory hypernociception.[11−14] Therefore, it is conceivable that **1** exerts its antinociceptive activity through prevention of oxidative imbalance caused by inflammatory agents such as carrageenin.

One question that emerges from our results is, what is the relationship between inhibition of IL-1β production and inhibition of oxidative stress by **1**? There is a great body of evidence that these events are interconnected. For instance, cytokines including TNFα and IL-1β can induce the production of H_2O_2 and superoxide, while H_2O_2 and superoxide induce the activation of NFκB, which upregulates cytokine production.[24] Moreover, inhibition of oxidative stress by treatment with a SOD mimetic inhibits carrageenin-induced hypernociception, which was associated with inhibition of cytokine production in the paw.[11] Thus, the inhibitory effect of **1** on IL-1β production might be a consequence of inhibition of oxidative stress, although reduction in IL-1β might produce an impact on oxidative stress.

Quercetin (1) Showed No Muscle-Relaxing or Sedative Effects. Intraperitoneal treatment with 100 mg/kg of **1** given 3 h 30 min beforehand did not alter the motor response of the test animals ($n = 6$). This time point was based on the 30 min of pretreament plus 3 h until mechanical hypernociception measurement. The vehicle control response in the Rotarod test was 180 s vs 180 s of **1**-treated animals (data not shown). These results support the notion that quercetin is diminishing the nociceptive threshold induced by inflammation and not that the mice are incapable of responding because of muscle-relaxing or sedative effects.

Quercetin (1) Did Not Exhibit Any Effect in the Hot-Plate Test in Naive Mice. Mice were treated with **1** (100 mg/kg, ip route) or morphine hydrochloride (8 mg/kg, ip route), and the thermal hypernociception was evaluated before and 30 and 60 min after treatment (data not shown). Morphine hydrochloride treatment increased the thermal threshold as expected because of its central analgesic effects. On the other hand, **1** did not alter the thermal threshold of mice (data not shown). This result further supports a specific effect of quercetin upon inflammatory pain and is in contrast with a study showing a slight analgesic effect of **1** in the tail-flick test in a naloxone-sensitive manner.[4] This difference might be related to the test used since the hot plate is considered a supraspinal modulated test, whereas the tail-flick method has more spinal reflex influence.[25]

In conclusion, the present study has further analyzed the antinociceptive activity of **1** upon inflammatory pain and has provided novel evidence for its mechanism of action. The antinociceptive mechanisms of action of **1** may depend on (a) opioid-like effects;[4] (b) activation of GABA and serotonin receptors, and nitric oxide production;[3] (c) inhibition of cytokine production (present study); and (d) inhibition of oxidative stress (present study). Therefore, quercetin is a commonly available plant natural compound with promising antinociceptive activity that merits further preclinical and clinical investigation.

Experimental Section

Chemicals. The following materials were obtained from the sources indicated. The National Institute for Biological Standards and Control (NIBSC, South Mimms, Hertfordshire, UK) provided recombinant murine TNFα and IL-1β and reagents for ELISA. Recombinant murine CXCL1 was purchased from PeproTech Inc. (Rocky Hill, NJ), acetic acid from Mallinckrodt Baker, S.A. (Mexico City, Mexico), carrageenin from FMC Corporation (Philadelphia, PA), quercetin (**1**) at 95% purity from Acros (Pittsburgh, PA), phenyl-*p*-benzoquinone from Sigma (St. Louis, MO), formalin from Merck (Darmstadt, Germany), and morphine hydrochloride from Crystalia (Campinas, SP, Brazil).

Animals. Male Swiss mice (25−30 g) from the University of Sao Paulo, Ribeirão Preto, Sao Paulo, 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 and kept at 21 °C. All behavioral testing was performed between 9 a.m. and 5 p.m. in a temperature-controlled room. Animal care and handling procedures were approved by the Ethics Committee of the Faculty of Pharmaceutical Sciences of Ribeirao Preto (University of Sao Paulo; protocol no. 04.1.994.53.2). All efforts were made to minimize the number of animals used and their suffering.

Nociception Tests. Electronic Pressure-Meter Test. The term "hypernociception" is used rather than hyperalgesia or allodynia to define the decrease in the nociceptive withdrawal threshold of animals.[26] Mechanical hypernociception was tested in mice as previously reported.[27] 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; IITC Life Science, Woodland Hills, CA) adapted with a 0.5 mm^2 polypropylene tip. 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 g) calculated by subtracting the zero-time mean measurements from the mean measurements 3 h after stimulus. Withdrawal threshold was 9.2 ± 0.5 g (mean ± SEM; $n = 30$) before injection of the hypernociceptive agents (e.g., cytokines or carrageenin).

Writhing Response Tests. The phenyl-*p*-benzoquinone (PBQ)- and acetic acid-induced writhing models were performed as previously described.[15] PBQ (diluted in DMSO 2%/saline, 1890 μg/kg), acetic acid (0.6% v/v, diluted in saline, 10 mL/kg), or vehicle was injected into the peritoneal cavities of mice pretreated with quercetin (**1**) (3−100 mg/kg, ip route). Each mouse was placed in a large glass cylinder, and the intensity of nociceptive behavior was quantified by counting the total number of writhes 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 writhing score over 20 min. Different individuals administered each test, prepared solutions to be injected, and performed the injections.

Formalin Test. The number of paw flinches and time spent licking the paw were determined between 0 and 30 min after intraplantar injection of 25 μL of formalin 0.1%, as previously described.[28] 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.[28]

Hot-Plate Test. Mice were placed in a 10 cm wide glass cylinder on a hot plate (IITC Life Science Inc. Woodland Hills, CA) maintained at 55 °C. Two control latencies at least 10 min apart were determined for each mouse. The normal latency (reaction time) was 5−9 s. The latency was also evaluated 30 and 60 min after test compound administration. The reaction time was scored when the animal jumped or licked its paws. A maximum latency (cutoff) was set at 30 s to avoid tissue damage.

Myeloperoxidase (MPO) Assay. The MPO kinetic−colorimetric assay was used to evaluate the leukocyte migration to the subcutaneous plantar tissue of the mouse hind paw.[21] Samples of subcutaneous plantar tissue were collected in 50 mM K_2HPO_4 buffer (pH 6.0) containing 0.5% hexadecyl trimethylammonium bromide (HTAB) and kept at −80 °C until use. Samples were homogenized using a Polytron (PT3100), centrifuged at 16.100*g* for 4 min, and the resulting supernatant was assayed spectrophotometrically for MPO activity determination at 450 nm (Spectra max), with three readings in 1 min. The MPO activity of the samples was compared to a standard curve of neutrophils. Briefly, 10 μL of sample was mixed with 200 μL of 50 mM phosphate buffer at pH 6.0, containing 0.167 mg/mL *o*-dianisidine dihydrochloride and 0.0005% hydrogen peroxide.

Measurement of Motor Performance. In order to discard possible nonspecific muscle relaxant or sedative effects of quercetin (**1**), mice motor performance was evaluated on the Rotarod test.[29] The apparatus consists 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 180 s. Animals were treated with vehicle (Tween 80 20% in saline) or **1** (30 mg/kg, po) 3 h 30 min before testing. The cutoff time used was 180 s.

Paw Edema Test. The volume of the mice paw was measured with a calliper (Ugo Basil, Italy) before (Vo) the intraplantar stimulus with carrageenan and 30, 60, 120, and 180 min after (VT).[29] The amount of paw swelling was determined for each mouse, and the difference between VT and Vo was taken as the edema value (edema mm^{-2}/paw).

Antioxidant Test. GSH is part of the endogenous antioxidant system, and its measurement is a parameter of oxidative stress.[21] The GSH skin levels were determined using a fluorescence assay as previously described.[21] The total mice hind paw plantar skin of five mice were pooled (1:3 dilution) and homogenized in 100 mM NaH_2PO_4 (pH 8.0) containing 5 mM EGTA using a Polytron (PT3100). Whole homogenates were treated with 30% trichloroacetic acid and centrifuged at 1940*g* for 6 min, and the fluorescence of the resulting supernatant was measured in a Hitachi F-4500 fluorescence spectrophotometer. Briefly, 100 μL of sample was mixed with 1 mL of 100 mM NaH_2PO_4 (pH 8.0) containing 5 mM EGTA and 100 μL of OPT (1 mg/mL in methanol). The fluorescence was determined after 15 min ($\lambda_{exc} = 350$ nm; $\lambda_{em} = 420$ nm). The standard curve was prepared with 0−40 μM GSH, and the results are presented as nM of GSH per mg of skin.

Cytokine Measurement. Mice were treated with vehicle or quercetin (**1**) (100 mg/kg, ip) 30 min before carrageenin (100 μg/paw) stimulus. Three hours after the injection of carrageenin, mice were terminally anesthetized, and the skin tissues were removed from the injected and control paws (saline and naive). The samples were homogenized in 500 μL of buffer containing protease inhibitors, and IL-1β, TNFα, and CXCL1 levels were determined as described previously[30] by an enzyme-linked immunosorbent assay (ELISA). The results are expressed as picograms (pg) of cytokine/paw. As a control, the concentrations of these cytokines were determined in animals injected with saline.

Experimental Protocols. Mice received oral (30, 100, and 300 mg/kg) or intraperitoneal (ip, 3, 10, 30, and 100 mg/kg) treatment with quercetin (**1**) or vehicle (20% Tween 80 in saline) 30 min before inflammatory stimulus. The doses of inflammatory stimuli were determined previously in our laboratory in pilot studies and based on previous work.[8,15,30,31] Mechanical hypernociception was evaluated 3 h after TNFα (300 pg/paw), CXCL1 (20 ng/paw), PGE_2 (100 ng/paw), or dopamine (10 μg/paw) injection. For carrageenin (100 μg/paw) stimulus, mechanical hypernociception and paw edema were evaluated at indicated time points (30 min to 5 h). All inflammatory stimuli induced only ipsilateral (in the paw the stimulus was injected) mechanical hypernociception. IL-1β, TNFα, and CXCL1 levels were evaluated 3 h after carrageenin (100 μg/paw) injection. GSH activity was evaluated 2 h after carrageenin (100 μg/paw) injection. Myeloperoxidase activity was evaluated 3 h after carrageenin (100 μg/paw), TNFα (300 pg/paw), or CXCL1 (20 ng/paw) injection. The writhing response was evaluated for 20 min after ip injection of acetic acid or phenyl-*p*-benzoquinone. The paw flinching and licking nociceptive responses were quantified for 30 min after formalin injection.

Statistical Analysis. Results are presented as mean ± SEM of experiments made on 5 (Figures 2−5) or 7 (Figure 1) animals per group and are representative of two separate experiments. Differences between groups were evaluated by analyses of variance (one-way ANOVA) followed by the Tukey test. Statistical differences were considered to be significant at $p < 0.05$.

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Supporting Information Available: Figures showing additional experiments conducted on **1** are available free of charge via the Internet at http://pubs.acs.org.

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