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The superoxide anion donor, potassium superoxide, induces pain and inflammation in mice through production of reactive oxygen species and cyclooxygenase-2

N.A. Maioli[1], A.C. Zarpelon[1], S.S. Mizokami[1], C. Calixto-Campos[1], C.F.S. Guazelli[1], M.S.N. Hohmann[1], F.A. Pinho-Ribeiro[1], T.T. Carvalho[1], M.F. Manchope[1], C.R. Ferraz[1], R. Casagrande[2] and W.A. Verri Jr.[1]

[1]Departamento de Patologia, Centro de Ciências Biológicas, Universidade Estadual de Londrina, Londrina, PR, Brasil
[2]Departmento de Ciências Farmacêuticas, Centro de Ciências da Saúde, Hospital Universitário, Universidade Estadual de Londrina, Londrina, PR, Brasil

Abstract

It is currently accepted that superoxide anion ($O_2^{\bullet-}$) is an important mediator in pain and inflammation. The role of superoxide anion in pain and inflammation has been mainly determined indirectly by modulating its production and inactivation. Direct evidence using potassium superoxide (KO_2), a superoxide anion donor, demonstrated that it induced thermal hyperalgesia, as assessed by the Hargreaves method. However, it remains to be determined whether KO_2 is capable of inducing other inflammatory and nociceptive responses attributed to superoxide anion. Therefore, in the present study, we investigated the nociceptive and inflammatory effects of KO_2. The KO_2-induced inflammatory responses evaluated in mice were: mechanical hyperalgesia (electronic version of von Frey filaments), thermal hyperalgesia (hot plate), edema (caliper rule), myeloperoxidase activity (colorimetric assay), overt pain-like behaviors (flinches, time spent licking and writhing score), leukocyte recruitment, oxidative stress, and cyclooxygenase-2 mRNA expression (quantitative PCR). Administration of KO_2 induced mechanical hyperalgesia, thermal hyperalgesia, paw edema, leukocyte recruitment, the writhing response, paw flinching, and paw licking in a dose-dependent manner. KO_2 also induced time-dependent cyclooxygenase-2 mRNA expression in the paw skin. The nociceptive, inflammatory, and oxidative stress components of KO_2-induced responses were responsive to morphine (analgesic opioid), quercetin (antioxidant flavonoid), and/or celecoxib (anti-inflammatory cyclooxygenase-2 inhibitor) treatment. In conclusion, the well-established superoxide anion donor KO_2 is a valuable tool for studying the mechanisms and pharmacological susceptibilities of superoxide anion-triggered nociceptive and inflammatory responses ranging from mechanical and thermal hyperalgesia to overt pain-like behaviors, edema, and leukocyte recruitment.

Key words: Superoxide anion; Potassium superoxide; Pain; Inflammation; Oxidative stress

Introduction

Inflammation-related events such as edema, leukocyte recruitment, and pain are important for the protection of organisms, but excessive and persistent inflammation leads to tissue damage, chronic pain, and organ dysfunction. Therefore, knowledge of inflammation mechanisms is essential to treat inflammatory diseases and to promote host protection and homeostasis (1).

Oxidative stress is an important component of pain and inflammation. Similarly, antioxidants such as the flavonoid, quercetin, inhibit overt pain-like behavior, carrageenan-induced mechanical hyperalgesia, and paw edema by diminishing interleukin-1β production and reducing glutathione (GSH) depletion (2). Quercetin also inhibits leukocyte recruitment (3) and acetic acid-induced colitis in mice (4). This is a consistent effect observed with antioxidants (5,6). Quercetin is considered to be a standard antioxidant flavonoid, because it presents all the structural groups related to antioxidant activity possessed by this class of molecules (2-4).

The role of reactive oxygen species (ROS) in pain and inflammation has been investigated *in vivo*. For instance, the superoxide dismutase (SOD) mimetics, M40403 and

Correspondence: W.A. Verri Jr.: <waldiceujr@yahoo.com.br> and/or <waverri@uel.br>.

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TEMPOL, inhibit carrageenan-induced thermal hyperalgesia, paw edema, and cytokine production by preventing the superoxide-induced nitration of SOD (7,8). Similarly, SOD inhibition by diethyldithiocarbamic acid increases carrageenan-induced paw edema and thermal hyperalgesia (8). The generation of peroxynitrite by the co-administration of pyrogallol (superoxide anion generator) and of a nitric oxide donor (SNAP) also induces thermal hyperalgesia and paw edema (8).

The main inflammatory source of superoxide anion is the complex of phagocytic NADPH oxidase enzymes. Production of superoxide anion and its derivatives is related to opioid tolerance, chemotherapy-induced pain, neuropathic pain, neurogenic pain, and inflammatory pain (9).

Despite current evidence regarding the crucial role of superoxide in pain and inflammation, to our knowledge, there are limited experimental approaches for studying the induction of pain and inflammation directly by superoxide anion. There is evidence that potassium superoxide (KO_2), a superoxide anion donor, induces thermal hyperalgesia in the Hargreaves method at 20 min (7) and 60 min (10) after its injection into the paw. However, to further support the use of KO_2 for investigating a wide range of superoxide anion-induced inflammatory and pain responses, it remains to be determined whether KO_2 induces additional nociceptive behaviors such as mechanical hyperalgesia and overt pain-like behaviors as well as inflammatory responses such as edema and leukocyte recruitment. In this context, we investigated KO_2 as a trigger for pain and inflammation in mice and the susceptibility of these responses to the analgesic opioid, morphine, the antioxidant flavonoid, quercetin, and the cyclooxygenase-2 (COX-2) selective inhibitor, celecoxib.

Material and Methods

Animals

Male Swiss mice (25 ± 5 g) from Universidade Estadual de Londrina were housed in standard plastic cages with free access to food and water, with a 12:12-h light/dark cycle, at 21°C. Care and handling procedures for the animals were in accordance with the International Association for the Study of Pain (IASP) guidelines and with the approval of the Ethics Committee of the Universidade Estadual de Londrina (process #71.20 12.68).

Drugs and reagents

KO_2 (96.5%) was purchased from Alfa Aesar (USA). Quercetin at 95% purity was purchased from Acros (USA). Morphine, naloxone, hexadecyl trimethyl ammonium bromide, *o*-dianisidine dihydrochloride, GSH, EDTA sodium salt, ferric chloride hexahydrate, 2,4,6-tripyridyl-s-triazine, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonate (ABTS), and 6-hydroxy-2,5,7,8-tetramethylchroman-2-11 carboxylic (Trolox) were purchased from Sigma (USA).

Experimental protocols

The parameters evaluated were mechanical hyperalgesia; thermal hyperalgesia; paw edema; paw flinches; paw licking; writhing response; total counts of leukocytes, mononuclear cells, and neutrophils; superoxide anion production; pharmacological susceptibility to morphine, quercetin, and celecoxib; oxidative stress methods (ferric-reducing ability potential [FRAP], ABTS, and GSH assays); and quantitative polymerase chain reaction (qPCR) for COX-2 mRNA expression. For mechanical hyperalgesia, thermal hyperalgesia, and paw edema, KO_2 was administered by the intraplantar (*ipl*) route at doses of 3-100 μg/paw in a final volume of 25 μL sterile saline, followed by evaluation after 0.5-7 h. Paw flinches and time spent licking the paw were determined between 0 and 30 min after *ipl* injection of KO_2 (1-30 μg/paw), and the total number of writhings was determined between 0 and 20 min after intraperitoneal (*ip*) injection of KO_2 (30-1000 μg/cavity, in a final volume of 100 μL of sterile saline). Total counts of leukocytes, mononuclear cells, and neutrophils were evaluated 6 h after *ip* injection of KO_2 (10-100 μg/cavity). Superoxide anion levels in KO_2 saline solution were determined by reduction of nitrobluetetrazolium (NBT) *in vitro*.

The pharmacological susceptibility of KO_2-induced nociception was determined using the opioid morphine, the antioxidant quercetin, and the COX-2 selective inhibitor celecoxib, as follows. Morphine: mice were treated with morphine (2-12 μg/paw, *ipl*, 20 μL, 30 min before measurement) and the opioid receptor antagonist naloxone (30 μg/paw, *ipl*, 1 h before morphine) or its vehicle. Quercetin: mice were treated with quercetin (10-100 mg/kg, *ip*) 30 min before KO_2 injection (30 μg/paw, *ipl*). Total counts of leukocytes, mononuclear cells, and neutrophils (6 h after stimulus injection), and antioxidant status (FRAP, ABTS, and GSH assays, 3 h after stimulus) were evaluated in animals pretreated with quercetin (100 mg/kg, *ip*), 30 min before *ip* or *ipl* injection of KO_2 (30 μg/cavity), respectively. Celecoxib: mice were treated with celecoxib (30 mg/kg, *ip*) 1 h before KO_2 injection (30 μg/paw, *ipl* or *ip*, and 1 mg/mice), and the parameters evaluated were mechanical hyperalgesia, thermal hyperalgesia, paw flinches, paw licking, and the writhing response. Whether KO_2 injection induced time-dependent (0.5-7 h) COX-2 mRNA expression by qPCR was also determined. Results are reported as means ± SE of measurements of 6 mice/group per experiment, and are representative of two separate experiments. It is noteworthy that different investigators prepared the solutions, administered them, and performed the testing procedures.

Electronic pressure meter test

Mechanical hyperalgesia was assessed as previously

reported (11). In a quiet room, mice were placed in acrylic cages ($12 \times 10 \times 17$ cm) with wire grid floors, 30 min before testing. The test consisted of evoking a hind paw reflex with a hand-held force transducer (electronic anesthesiometer; Insight Equipamentos, Brazil) adapted with a 0.5 mm^2 polypropylene tip. The investigator was trained to apply the tip perpendicularly to the central area of the hind paw with a gradual increase in pressure. The end point was characterized by the removal of the paw followed by clear flinching movements, and the intensity of pressure was recorded automatically. The animals were tested before (basal) and after treatment and stimuli, and the value for each interval was an average of 3 measurements. The results are reported as the change (Δ) in withdrawal threshold (in g), calculated by subtracting the basal mean measurements from the mean measurements obtained at 0.5, 1, 3, 5, or 7 h after *ipl* injection of KO_2 (3-100 μg/paw). The dose of KO_2 of 30 μg/paw was selected for pharmacological testing.

Hot plate test

Mice were placed in a 10 cm wide glass cylinder on a hot plate (EFF 361, Insight Equipamentos) maintained at 55°C. Two control latencies at least 10 min apart were determined for each mouse. The normal latency (reaction time) was 12-20 s. The latency was also evaluated 0.5, 1, 3, 5, and 7 h 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 (12). A dose-response curve was performed using KO_2 at doses of 3-100 μg/paw, and 30 μg/paw was selected for pharmacological testing.

Writhing response tests

Each mouse was placed in a large glass cylinder and the intensity of nociceptive behavior was quantified by counting the total number of writhings (contraction of the abdominal muscle together with a stretching of hind limbs) occurring between 0 and 20 min after *ip* injection of KO_2. The intensity of nociceptive behavior was expressed as the cumulative number of writhings over 20 min. A dose-response curve was performed using KO_2 at doses of 30-1000 μg/cavity (*ip*), and the dose of 1000 μg/mouse was selected for pharmacological testing.

Paw flinches and time spent licking test

The number of paw flinches and time spent licking the stimulated paw were determined between 0 and 30 min after *ipl* injection of KO_2. Results are reported as the cumulative number of paw flinches and time spent licking the paw over 30 min. A dose-response curve was performed using KO_2 at doses of 1-30 μg/paw, and 30 μg/paw was selected for pharmacological testing.

Paw edema

Paw edema was measured using an analog caliper (Digmatic Caliper, Mitutoyo Corporation, Japan). Values of paw edema are reported as the difference between the paw thickness measured before (basal) and after induction of paw inflammation in millimeters. A dose-response curve was performed using KO_2 at doses of 3-100 μg/paw, and 30 μg/paw was selected for pharmacological testing.

Myeloperoxidase (MPO) activity

The MPO kinetic-colorimetric assay was performed as described elsewhere (11) to verify leukocyte migration to the subcutaneous plantar tissue of the mouse hind paw. Seven hours after KO_2 injection, the hind paw tissue of mice was collected and homogenized using Tissue-Tearor (Biospec, USA), centrifuged at 16,100 *g* for 4 min, and the resulting supernatant was assayed spectrophotometrically for MPO activity determination at 450 nm (Multi Scan Go Thermo Scientific, USA). The MPO activity of the samples was compared to a standard curve of neutrophils, and the results are reported as MPO activity (number of neutrophils $\times 10^3$/mg of tissue). A dose-response curve was performed using KO_2 at doses of 3-100 μg/paw, and 30 μg/paw was selected for pharmacological testing.

Leukocyte recruitment to the peritoneal cavity

Peritoneal cavities were washed with 1 mL of phosphate-buffered saline (PBS) containing EDTA (37.2 mg/100 mL saline). Total leukocyte counts were performed in a Neubauer chamber after dilution in Turk's solution (2% acetic acid, v/v), and differential cell counts were performed using the Fast Panoptic Kit for histological analysis (Laborclin, Brazil). Results are reported as number of cells per cavity ($\times 10^6$). Leukocyte recruitment was determined 6 h after KO_2 injection. Total and differential cell counts were both performed under a light microscope (400× magnification, Olympus Optical Co., Germany) (12). A dose-response curve was calculated using KO_2 at doses of 10-100 μg/paw, and 30 μg/paw was selected for pharmacological testing.

FRAP and ABTS assays

Paw skin samples were collected and immediately homogenized with 500 μL of 1.15% KCl, and centrifuged (10 min × 200 *g* × 4°C). The ability of a sample to resist oxidative damage was determined using FRAP and ABTS assays (13). 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 absorbance measured at 595 nm. For the ABTS assay, the ABTS solution was diluted with PBS at pH 7.4 at 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 reported as Trolox equivalents per milligram of tissue weight in both assays.

GSH measurement

Paw skin sample was collected and maintained at –80°C for at least 48 h, then the sample was homogenized with 200 μL of 0.02 M EDTA; the homogenate was mixed with 25 μL of 50% trichloroacetic acid and homogenized three times for 15 min. The mixture was then centrifuged (15 min × 1500 *g* × 4°C) and the 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, absorbance was measured at 412 nm against a blank reagent with no supernatant. A standard curve was calculated using standard GSH, and the results are reported as GSH per milligram protein (14). For protein determination, 60 μL of supernatant were mixed with 60 μL of freshly prepared copper reagent. After 10 min, 180 μL of Folin solution was added. The resulting solution was incubated at 50°C for 10 min, the absorbance was measured at 660 nm, and the results were equated to a standard curve of bovine serum albumin.

In vitro superoxide anion production assay

An NBT reduction assay was carried out to evaluate the ability of KO_2 to release superoxide anion at varied time points after its dilution in saline (30 μg, 25 μL). An equimolar solution of KOH diluted in saline was used as control. KO_2 or KOH solutions were mixed with 100 μL of NBT reagent (1 mg/mL) at the indicated time points after dissolution. After 15 min of incubation at room temperature, the supernatants were carefully removed. The formazan precipitates were solubilized by addition of 120 μL of KOH (2 M) and 120 μL of DMSO, and absorbance was read at 600 nm (15).

qPCR

Paw skin samples were homogenized in TRIzol reagent (Life Technologies, USA), and total RNA was isolated according to the manufacturer's instructions. RNA purity was confirmed by the 260/280 ratio. RT-PCR and qPCR were performed using GoTaq 2-Step RT-qPCR System (Promega, USA) following the manufacturer's instructions. Complementary DNA was reversely transcribed from 2 mg of total RNA, and qPCR was performed for 50 cycles on a LightCycler Nano Instrument (Roche, Switzerland). Primer sequences were as follows: *Cox-2*, forward: 5′-GTGGAAAAACCTCGTCCAGA-3′, reverse: 5′-GCTCGGCTTCCAGTATTGAG-3′; and *Gapdh*, forward: 5′-CATACCAGGAAATGAGCTTG-3′, reverse: 5′-ATGACATCAAGAAGGTGGTG-3′.

Statistical analysis

Results are reported as means ± SE of measurements made on 6 mice/group per experiment and are representative of 2 separate experiments. Two-way analysis of variance (ANOVA) was used to compare the groups and doses at all times (curves). The analyzed factors were treatment, time, and time *vs* treatment interaction. When there was a significant time *vs* treatment interaction, one-way ANOVA followed by Tukey's *t*-test was performed for each time. On the other hand, when the nociceptive responses were presented as total values at the indicated time period, the differences between responses were evaluated by one-way ANOVA followed by Tukey's *t*-test. Statistical differences were considered to be significant at $P<0.05$.

Results

Superoxide anion donor KO_2 induced mechanical hyperalgesia, thermal hyperalgesia, paw edema, and MPO activity

KO_2 was administered *ipl* at doses of 3-100 μg/paw in a final volume of 25 μL of sterile saline. Mechanical hyperalgesia (Figure 1A), thermal hyperalgesia (Figure 1B), and paw edema (Figure 1C) were evaluated 0.5-7 h after stimulus. All doses of KO_2 induced statistically significant mechanical hyperalgesia compared to the control group (saline) at all time points, except the dose

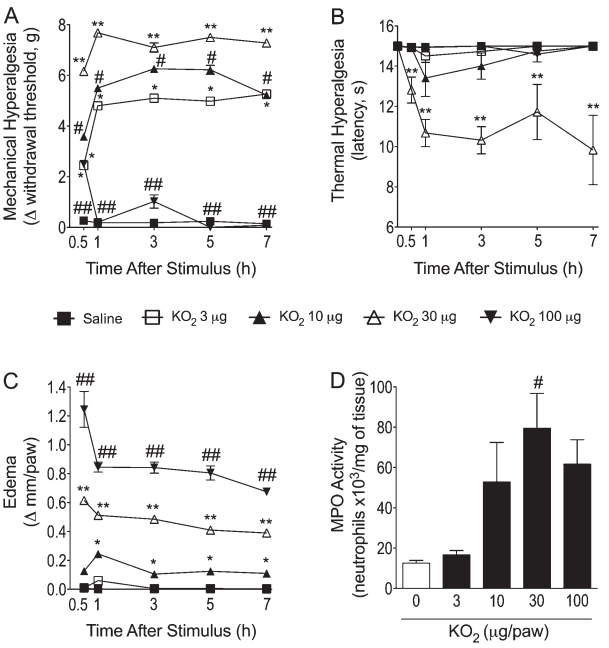

Figure 1. Intraplantar (*ipl*) injection of KO_2 induced hyperalgesia, edema, and neutrophil recruitment. Mice received KO_2 (3-100 μg, 25 μL, *ipl*) or saline injection. The intensity of mechanical hyperalgesia (*A*), thermal hyperalgesia (*B*), and the paw edema (*C*) were measured 0.5-7 h after stimulus, and myeloperoxidase (MPO) activity at 7 h. Data are reported as means ± SE. *$P<0.05$ *vs* saline control; #$P<0.05$ *vs* 3 μg; **$P<0.05$ *vs* 10 μg; ##$P<0.05$ *vs* 30 μg (one-way ANOVA followed by Tukey's *t*-test).

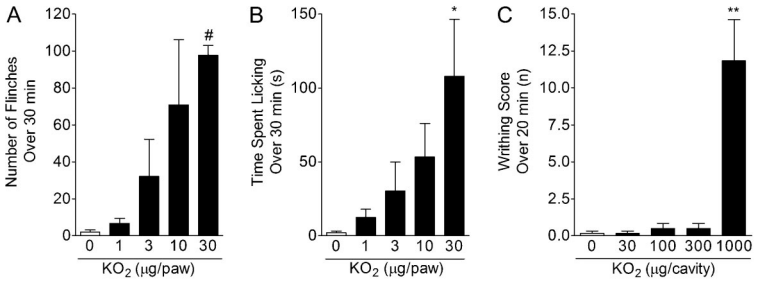

Figure 2. KO_2 induced overt pain-like behavior. The number of paw flinches (*A*), and the time spent licking the paw (*B*) were evaluated during 30 min after KO_2 (1-100 μg, *ipl*) or saline injection. The writhing score was evaluated 20 min after KO_2 (30-1000 μg, *ip*) or saline injection. Data are reported as means ± SE. *P<0.05 *vs* saline control; #P<0.05 *vs* 1 μg; **P<0.05 *vs* 300 μg (one-way ANOVA followed by Tukey's *t*-test).

of 100 μg/paw (Figure 1A). KO_2 at 30 μg/paw induced statistically significant mechanical hyperalgesia compared to the other doses (Figure 1A). KO_2 induced mechanical hyperalgesia in a bell-shaped curve, achieving the maximal effect with 30 μg/paw and almost no mechanical hyperalgesia with 100 μg/paw. Regarding thermal hyperalgesia, doses of 3, 10, and 100 μg/paw of KO_2 showed similar thresholds (Figure 1B). Only the dose of 30 μg/paw induced a statistically significant reduction in thermal threshold when compared to the control group at all evaluated times (Figure 1B). KO_2 induced a dose-dependent increase in paw edema. KO_2 did not induce paw edema at 3 μg/paw, the dose of 10 μg/paw induced paw edema at 0.5 and 1 h, and doses of 30 and 100 μg/paw induced paw edema at all time points (Figure 1C). Only the dose of 30 μg/paw of KO_2 induced a significant increase in MPO activity (Figure 1D).

Superoxide anion induced dose-dependent pain-like behavior

KO_2 was administered at doses of 1-30 μg/paw, and the number of flinches and the time spent licking the paw were quantified (Figure 2, A and B). KO_2 induced a dose-dependent increase in the number of paw flinches and time spent licking the paw. Only the dose of 30 μg/paw induced statistically significant nociceptive responses compared to the vehicle control group. The writhing response was induced only by a dose of 1000 μg/cavity of KO_2 (Figure 2C).

Superoxide anion donor induced leukocyte recruitment

KO_2 (10-100 μg/cavity) was injected *ip* and peritoneal exudates were collected after 6 h, which is a standard time point for acute leukocyte recruitment to the peritoneal cavity (Figure 3A) (16). All doses induced significant increases in total leukocyte counts in a bell-shaped curve, with significant differences with the dose of 30 μg compared to 10 and 100 μg of KO_2. A bell-shaped curve also resulted with counts for total mononuclear cells (Figure 3B) and neutrophils (Figure 3C). Again, the maximal effect was observed with the dose of 30 μg of KO_2, which was selected for the leukocyte recruitment experiments in this study.

KO_2 in saline presented time-dependent decay of superoxide anion levels

An important point to be determined was whether KO_2 saline solution would present detectable superoxide anion, which was tested with KO_2 solution at 30 μg/25 μL, a concentration selected on the basis of the results of Figures 1-3. Saline solution did not reduce NBT, indicating that there was no detectable superoxide anion (Figure 4). KO_2 saline solution presented significant NBT reduction activity, indicating detectable superoxide anion levels at 1 and 5 min after solution preparation. Ten minutes after KO_2 saline solution preparation, all NBT reducing activity vanished, indicating that no superoxide anions were present. A KOH saline solution at an equimolar concentration of the KO_2 solution was tested

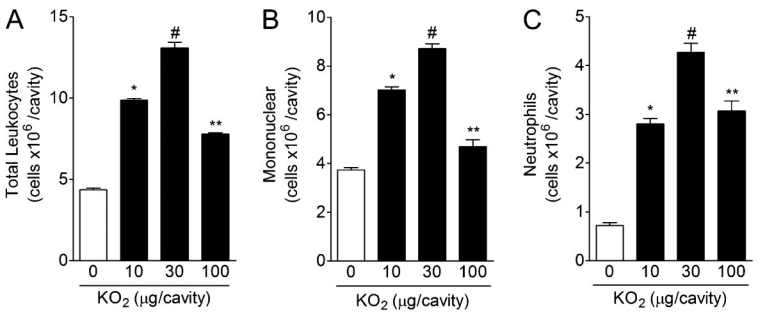

Figure 3. KO_2 induced leukocyte recruitment to the peritoneal cavity. The peritoneal cells were harvested 6 h after KO_2 (10-100 μg, *ip*) or saline injection. Total (*A*) and differential (*B* and *C*) cell counts were determined. Data are reported as means ± SE. *P<0.05 *vs* saline control; #P<0.05 *vs* 10 μg; **P<0.05 *vs* 30 μg (one-way ANOVA followed by Tukey's *t*-test).

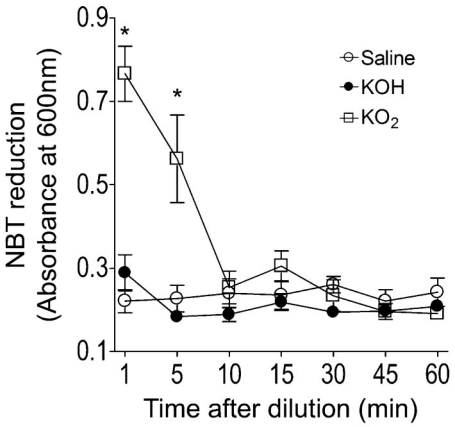

Figure 4. KO_2 dissolution in saline produced superoxide anion. KO_2 (30 μg, 25 μL of saline) or KOH (equimolar concentration to KO_2, 25 μL of saline) solutions were prepared and mixed with 100 μL of nitrobluetetrazolium (NBT) reagent at the indicated time points. Data are reported as means ± SE. *P<0.05 *vs* saline control and KOH solution (one-way ANOVA followed by Tukey's *t*-test).

as control. The KOH saline solution did not present detectable superoxide anion levels (Figure 4). These results demonstrated that KO_2 saline solution injected up to 5 min after its preparation presents significant amounts of superoxide anion, while the procedure for its injection into one group of mice takes approximately 3 min. Therefore, these data confirmed the successful administration of superoxide anion.

KO_2-induced hyperalgesia and pain-like behavior were inhibited by morphine treatment

Mice received 30 μg/paw of KO_2 and after 2 h were treated with morphine (opioid, 2-12 μg/paw) and, after an additional 1 h, mechanical (Figure 5A) and thermal (Figure 5B) hyperalgesia were evaluated. The dose of 2 μg morphine did not alter KO_2-induced mechanical hyperalgesia, while 6 μg morphine reduced mechanical hyperalgesia compared to the control group, and 12 μg morphine reduced mechanical hyperalgesia compared to all lower doses (Figure 5A). Morphine was also effective in reducing KO_2-induced thermal hyperalgesia at a dose of 12 μg (Figure 5B). Naloxone (1 mg/kg, *ip*, 30 min before morphine treatment) prevented the analgesic effect of morphine over KO_2-induced mechanical (Figure 5A) and thermal (Figure 5B) hyperalgesia. Morphine at a dose of 12 μg/paw (cotreatment) also inhibited KO_2-induced paw flinching (Figure 5C) and licking (Figure 5D) behaviors, and, again, naloxone treatment prevented the analgesic effect of morphine.

The standard antioxidant flavonoid, quercetin, inhibited KO_2-induced mechanical hyperalgesia, thermal hyperalgesia, and edema

Mice were treated with quercetin (10-100 mg/kg, *ip*)

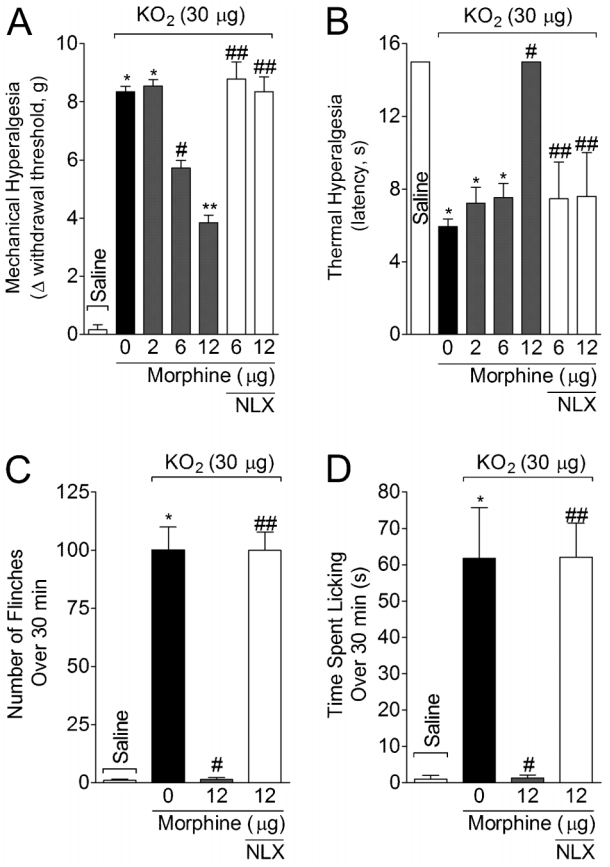

Figure 5. Morphine inhibited KO_2-induced hyperalgesia and overt pain-like behavior in a naloxone-sensitive manner. Mice were post-treated (*A* and *B*) with morphine (2-12 μg, *ipl*) or vehicle (saline) 45 min before the measurements. The nociceptive thresholds to mechanical (*A*) and thermal (*B*) stimuli were measured 3 h after KO_2 (30 μg/paw) injection. In another set of experiments, mice were pre-treated (*C* and *D*) with morphine (12 μg, *ipl*) or vehicle (saline) 45 min before KO_2 (30 μg/paw) or saline injection. The number of paw flinches (*C*), and the time spent licking the paw (*D*) were measured during 30 min. Treatment with naloxone is indicated as NLX (1 mg/kg, *ip*, 30 min before morphine injection). Data are reported as means ± SE. *P<0.05 *vs* saline control; #P<0.05 *vs* KO_2 control (black bars); **P<0.05 *vs* 6 μg morphine; ##P<0.05 *vs* 12 μg morphine (one-way ANOVA followed by Tukey's *t*-test).

1 h before *ipl* injection of KO_2 (Figure 6). Quercetin inhibited KO_2-induced mechanical hyperalgesia (Figure 6A), thermal hyperalgesia (Figure 6B), and paw edema (Figure 6C) in a dose-dependent manner. Thus, the dose of 100 mg/kg quercetin was selected for the experiments presented in Figures 7-9.

Quercetin inhibited KO_2-induced overt pain-like behavior, leukocyte recruitment to the peritoneal cavity, and oxidative stress

Quercetin inhibited KO_2-induced overt pain-like behaviors

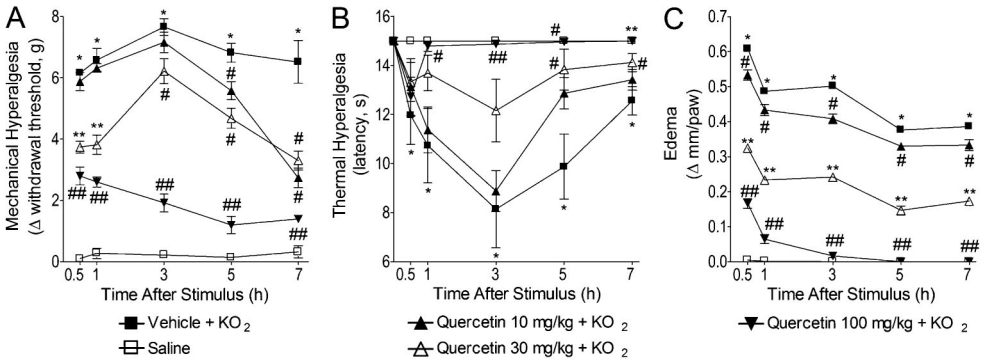

Figure 6. Quercetin inhibited the KO_2-induced mechanical hyperalgesia (*A*), thermal hyperalgesia (*B*), and edema (*C*) in a dose-dependent manner. Mice were treated with quercetin (10-100 mg/kg, *ip*) or vehicle (20% tween 80 in saline, 150 µL, *ip*) 1 h before KO_2 (30 µg/paw) or saline injection. Data are reported as means ± SE. *P<0.05 *vs* saline control; #P<0.05 *vs* KO_2 control; **P<0.05 *vs* 10 mg/kg; ##P<0.05 *vs* 30 mg/kg (one-way ANOVA followed by Tukey's *t*-test).

of paw flinching (Figure 7A), paw licking (Figure 7B), and abdominal writhing (Figure 7C). Quercetin also inhibited KO_2-induced recruitment of total leukocytes (Figure 8A), mononuclear cells (Figure 8B), and neutrophils (Figure 8C) to the peritoneal cavity of the mice. The *ipl* injection of KO_2 induced diminished ABTS (Figure 9A), FRAP (Figure 9B), and GSH levels (Figure 9C) in plantar skin samples. Quercetin inhibited a KO_2-induced decrease in ABTS (Figure 9A), as well as FRAP (Figure 9B) and GSH levels.

The COX-2 selective inhibitor, celecoxib, inhibited KO_2-induced nociception and COX-2 mRNA expression

Mice were treated with celecoxib (30 mg/kg, *ip*) (17) 1 h before *ipl* (30 µg/paw) or *ip* (1 mg/mice) injection of KO_2 (Figure 10). Celecoxib inhibited KO_2-induced mechanical hyperalgesia (Figure 10A), thermal hyperalgesia (Figure 10B), paw flinching (Figure 10C), paw licking (Figure 10D), abdominal writhing (Figure 10E), and COX-2 mRNA expression in paw skin samples (Figure 10F). Therefore, KO_2-induced nociception depends on COX-2.

Discussion

Superoxide anion has been established as an important mediator of pain and inflammation (6-9). Most of the evidence in this regard targeted superoxide anion metabolism to evaluate its inflammatory and nociceptive effects. An important approach to study the effects of superoxide anion would be to inject a superoxide anion donor such as KO_2. KO_2 injection induces thermal hyperalgesia in the Hargreaves test (7,10). In the present study, we provided evidence for the induction of varied nociceptive and inflammatory responses in mice by administering KO_2. The KO_2-induced nociceptive behaviors were responsive to treatment with the opioid receptor agonist, morphine, and COX-2 selective inhibitor, celecoxib (18,19). Furthermore, an antioxidant with analgesic and anti-inflammatory effects, quercetin (2,4), inhibited KO_2-induced pain, inflammation, and oxidative stress.

KO_2 has been used as a generator of superoxide anion *in vivo* (7,10). Superoxide anion is generated in normal aerobic metabolism by one-electron transfer to molecular oxygen (20), as well as by NADPH oxidase during inflammation *in vivo* (21). Superoxide anion is a

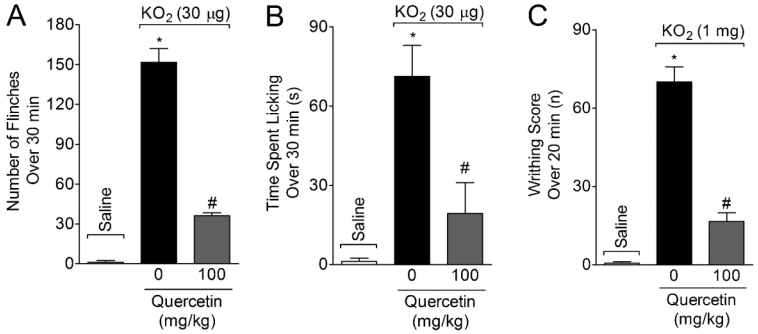

Figure 7. Quercetin inhibited the KO_2-induced overt pain-like behaviors. Mice were treated with quercetin (100 mg/kg, *ip*) or vehicle 1 h before stimuli. The number of paw flinches (*A*), and the time spent licking the paw (*B*) were quantified during 30 min after KO_2 (30 µg/paw) injection. The writhing score (*C*) was quantified during 20 min after KO_2 (1 mg, *ip*) injection. Data are reported as means ± SE. *P<0.05 *vs* saline control; #P<0.05 *vs* KO_2 control (one-way ANOVA followed by Tukey's *t*-test).

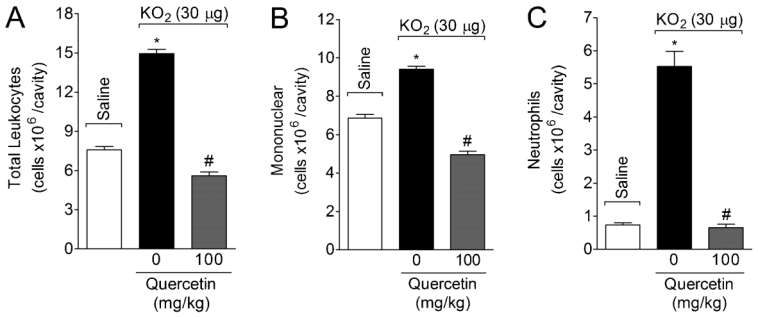

Figure 8. Quercetin inhibited the KO_2-induced leukocyte recruitment. Mice were treated with quercetin (100 mg/kg, *ip*) or vehicle 1 h before KO_2 (30 μg, *ip*) injection. The total number of leukocytes (*A*), mononuclear cells (*B*), and neutrophils (*C*) were determined 6 h after stimulus. Data are reported as means ± SE. *$P<0.05$ *vs* saline control; $^{\#}P<0.05$ *vs* KO_2 control (one-way ANOVA followed by Tukey's *t*-test).

precursor of additional ROS with enhanced toxicity, including hydroxyl radical, hypochlorous acid, and singlet oxygen (22). Nevertheless, the actions of superoxide anion are not solely related to deleterious effects, but rather it is also a signaling molecule. For instance, neuropathy- and inflammation-induced superoxide anion activate p38 mitogen-activated protein (MAP) kinase, which in turn activates the transcription factor NFκB, and JNK and ERK MAP kinases, which activate activating protein-1 (AP-1). NFκB and AP-1 increase the expression of pro-inflammatory molecules (1,10,23).

KO_2 injection is a simple procedure that can be reproduced in any laboratory, making it easy to access the mechanisms triggered by increased superoxide anion levels. It is essential to inject KO_2 solution up to 5 min after its preparation, since the levels of superoxide anion decay rapidly in aqueous solution. KO_2 induced pronounced inflammatory and nociceptive responses irrespective of the administration foci. Therefore, its action is not restricted to one tissue. This model of superoxide anion-dependent inflammation and pain exhibited dose-dependent effects, except in abdominal writhing behavior in which only the higher dose tested was capable of inducing this behavior. Moreover, the dose of KO_2 of 100 μg/paw did not induce further increases in mechanical and thermal hyperalgesia compared to the dose of 30 μg/paw. It is possible that 100 μg/paw induced an exaggerated inflammatory response. This condition is similar to sepsis, in which there is paralysis of neutrophil recruitment when the primary infectious foci are not under control and overt inflammation and infection become systemic (24). Accordingly, 100 μg/paw of KO_2 induced equal or even lower responses compared to 10 μg/paw with respect to MPO activity, total leukocytes, mononuclear cells, and neutrophil recruitment, while 30 μg/paw induced significant responses compared to 10 and 100 μg/paw. Considering that recruited neutrophils contribute to hyperalgesia (25-27), it is likely that reduced leukocyte recruitment with 100 μg compared to 30 μg of KO_2 also helps explain why 100 μg did not induce further increases in nociception. Another important feature of the model is its susceptibility to pre- and post-treatment, as observed for quercetin and celecoxib, and morphine, respectively.

In addition to KO_2 injection (7,10), previous models of ROS-induced inflammation and pain include inhibitors of hyperalgesia induced by mitochondrial electron transport complexes I (rotenone) and III (antimycin) (28), administration of the ROS donor tert-butyl hydroperoxide-induced hyperalgesia (29), and peroxynitrite-induced enhancement of carrageenan-induced paw edema and thermal hyperalgesia (8). The emerging literature demonstrated that modulation of superoxide anion using SOD mimetics (M40403, TEMPOL) and peroxynitrite levels using the peroxynitrite decomposition catalyst [FeTM-4-PyP(5+)] reduce inflammatory mechanical hyperalgesia (23), thermal hyperalgesia (7,8,10,30), paw flinching, paw licking (31), paw edema (8,10), and leukocyte recruitment (21). To our knowledge, this is the first study to demonstrate that superoxide anion donor KO_2 induces mechanical

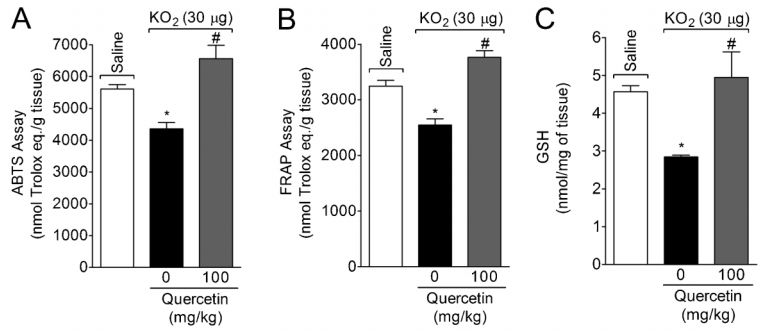

Figure 9. Quercetin inhibited KO_2-induced oxidative stress. Mice were treated with quercetin (100 mg/kg, *ip*) or vehicle 1 h before KO_2 (30 μg/paw) injection. The antioxidant capacity was measured by ABTS (*A*), FRAP (*B*), and GSH (*C*) assays, 3 h after stimulus injection. ABTS: free-radical scavenging ability; FRAP: ferric-reducing ability potential; GSH: glutathione. Data are reported as means ± SE. *$P<0.05$ *vs* saline control; $^{\#}P<0.05$ *vs* KO_2 control (one-way ANOVA followed by Tukey's *t*-test).

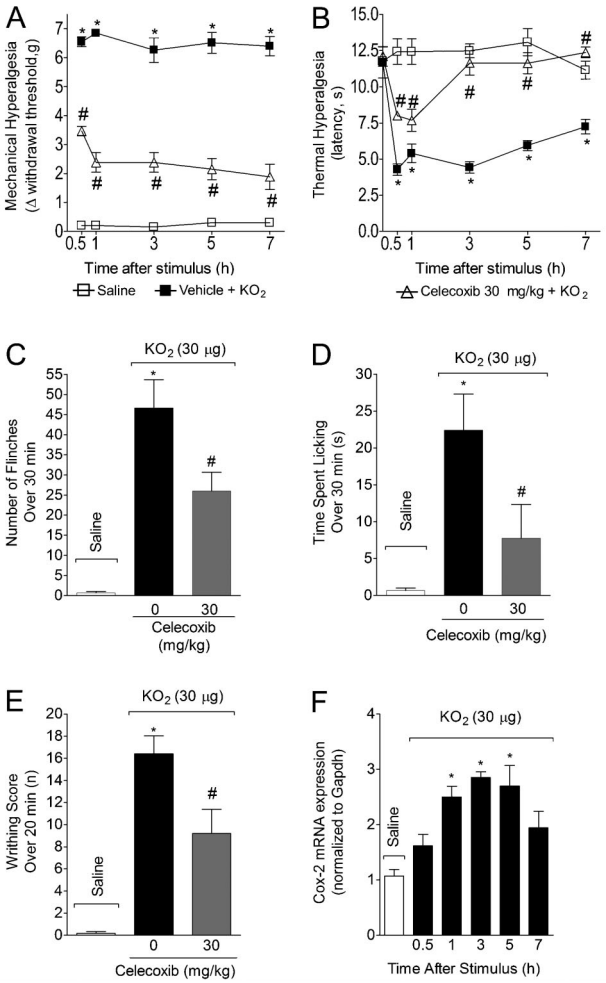

Figure 10. KO_2 induced COX-2-dependent nociception. Mice were treated with celecoxib (30 mg/kg, *ip*) or vehicle (saline) 1 h before *ipl* (30 μg, 25 μL) or *ip* (1 mg/mice) injection of KO_2. The intensity of mechanical hyperalgesia (*A*) and thermal hyperalgesia (*B*) were measured 0.5-7 h after stimulus, paw flinching (*C*) and licking (*D*) were evaluated during 30 min, and writhing response was evaluated during 20 min (*E*). COX-2 mRNA expression (*F*) was evaluated by qPCR 0.5-7 h after *ipl* injection of KO_2. Data are reported as means ± SE. *$P<0.05$ *vs* saline control; $^{\#}P<0.05$ *vs* vehicle control (0 mg/kg) (one-way ANOVA followed by Tukey's *t*-test).

hyperalgesia, thermal hyperalgesia in the hot plate test, paw edema, paw licking, abdominal writhing, and neutrophil recruitment. In agreement with the role of ROS in these responses and the present data, antioxidants such as quercetin inhibit carrageenan-induced mechanical hyperalgesia, thermal hyperalgesia in the hot plate test and paw edema, formalin-induced paw licking and flinching, acetic acid- and phenyl-p-benzoquinone-induced abdominal contortions, and carrageenan-induced MPO activity in paw skin, as well as neutrophil recruitment to the abdominal cavity (2,5,6). These data also justify the selection of quercetin in the present study. Quercetin is able to directly scavenge superoxide anion (32), as well as inhibit COX-2 mRNA expression and prostaglandin E_2 production (33). Therefore, this study did not exclude the possibility that quercetin could be acting by inhibiting COX-2 mRNA expression and prostaglandin E_2 production, in addition to its antioxidant effect.

Excessive superoxide anion production has been attributed to inhibition of mitochondrial manganese SOD (MnSOD), an enzyme that controls the levels of superoxide anion. Superoxide anion reacts with nitric oxide to generate peroxynitrite that, in turn, inactivates MnSOD by nitration of Tyr-34 in a manganese-catalyzed process. This is known as a ''feed-forward'' mechanism that allows further accumulation of superoxide anion and peroxynitrite (9). Despite the hypothesis that peroxynitrite mediates superoxide anion-induced hyperalgesia, there is evidence that nitric oxide and superoxide anion may operate independently of each other to induce pain (34).

Intrathecal administration of tert-butyl hydroperoxide (a ROS donor) increases the frequency and amplitude of spontaneous excitatory postsynaptic currents in neurons, which was inhibited by antagonists of non-N-methyl-D-aspartate glutamate, TRPA1 and TRPV1 receptors. These results indicate a neuronal effect of ROS by inducing glutamate release and TRPA1 and TRPV1 activity (29). Thus, ROS may act as neuronal activators, which lines up well with the immediate nociceptive responses observed by injection of KO_2, such as paw flinching, paw licking, and abdominal contortions. On the other hand, superoxide anion (from pyrogallol) and hydroxyl radical (from Fe-EDTA in hydrogen peroxide) did not induce ongoing activity or nociceptor sensitization to heat and mechanical stimuli (35), arguing against a direct neuronal effect of ROS. Considering that the effects of KO_2 include the induction of leukocyte recruiment, which depends on activation of tissue resident cells, the present data corroborate that ROS may trigger direct and indirect neuronal events (29,35).

In conclusion, the present data add to the literature regarding the inflammatory and nociceptive roles of superoxide anion and ROS, and demonstrate that KO_2 is a useful tool to trigger a great variety of superoxide anion-dependent inflammatory and nociceptive responses, which include thermal hyperalgesia in the Hargreaves test (7), mechanical hyperalgesia, thermal hyperalgesia in the hot plate test, paw edema, paw licking, paw flinching, abdominal writhing, and neutrophil recruitment (present data).

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