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ORIGINAL RESEARCH

Trans-Chalcone Reduces Inflammation and Pain Triggered by Superoxide Anion: Neuronal and Non-Neuronal Mechanisms

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Background: Potassium superoxide (KO_2), a superoxide anion donor, can be applied to induce reactive oxygen species (ROS) triggered pain and inflammation. *trans*-Chalcone (TC) is an atypical flavonoid because its molecular structure does not possess intrinsic antioxidant properties. This characteristic allows investigating the mechanisms of action of flavonoids excluding inherent chemical antioxidant effect. In the present study, we investigated the activity and mechanisms of TC in a model of inflammation and pain triggered by a superoxide anion donor, which to our knowledge have not been assessed yet.

Methods: Overt pain-like behavior, mechanical hyperalgesia, edema, leukocyte recruitment, oxidative stress markers, cytokine dosage by enzyme-linked immunosorbent assay (ELISA), nuclear factor kappa B (NF-κB) phosphorylation by Western blotting, mRNA expression by reverse transcription quantitative polymerase chain reaction (RT-qPCR), and neuronal activity by calcium levels were assessed. TC was administered orally 30 min before stimulation with KO_2, and a dose of 30 mg/kg was selected based on previous study.

Results: TC inhibited abdominal contortion, mechanical hyperalgesia, paw edema, and myeloperoxidase activity (an indirect marker of macrophage/neutrophil recruitment). TC induced antioxidant activity (assessed by ferric reducing ability and free radical scavenging), while reducing superoxide anion production and lipid peroxidation, at least in part, by upregulating *Nrf2* and downregulating $Gp91^{phox}$ and *Cox-2* mRNA expression. TC inhibited KO_2-induced NF-κβ phosphorylation as well as interleukin (IL)-1β, tumor necrosis factor (TNF)-α, IL-6, and IL-33 production. Finally, TC reduced the activation of transient receptor potential vanilloid 1 ($TRPV1^+$) and transient receptor potential ankyrin 1 ($TRPA1^+$) nociceptive neurons in the dorsal root ganglia.

Conclusion: These results demonstrate that the in vivo anti-inflammatory and analgesic activities of TC involve neuronal and non-neuronal mechanisms, and that non-antioxidant flavonoids are still biologically active.

Keywords: analgesic, potassium superoxide, TRPV1, TRPA1, nociception, flavonoids

Introduction

Reactive oxygen species (ROS), such as superoxide anion, are important regulators of both physiological and pathological conditions.[1] Under physiological conditions, they are necessary for cell signaling; however, under pathological conditions, they can be harmful, induce oxidative damage, and lead to disease and cell death.[2] Important pathological conditions such as atherosclerosis, chronic obstructive pulmonary disease, Alzheimer's disease, and cancer are, at least in part, oxidative damage-induced conditions.[3] Superoxide anion is an important ROS not only due to its activities, but also

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because it is a substrate to form other ROS such as hydroxyl radical and peroxynitrite.[4] Therefore, ROS mechanisms and therapies targeting ROS can be studied focusing on superoxide anion.

Potassium superoxide (KO_2) is a superoxide anion donor known to induce inflammation and pain. Superoxide anion activates varied cell types ranging from endothelial cells and immune cells to neurons. Endothelial cells are essential in the regulation of vascular permeability that will lead to plasma exudation in inflammation as well as by expressing adhesion molecules to allow leukocyte recruitment. Superoxide anion activates protein kinase C that phosphorylates the pro-inflammatory transcription factor nuclear factor κB (NF-κB) in endothelial cells.[5] In macrophages, superoxide anion, but not hydrogen peroxide or hydroxyl radical, is necessary for TNFα-induced activation of NF-κB.[6] Macrophages are tissue resident immune cells or even recruited monocytes that differentiate into macrophages, which produce other inflammatory molecules depending on the activation of NF-κB.[7–9] Among these inflammatory molecules, there are cytokines such as tumor necrosis factor (TNF)-α,[10] interleukin (IL)-1β,[9] IL-33,[11] and IL-10.[12] These cytokines contribute to orchestrating the inflammatory response by recruiting leukocytes and causing nociceptive neuronal sensitization.[13]

Cytokines contribute to pain by inducing the expression and activation of cyclooxygenase-2 (COX-2), a core enzyme in the production of prostaglandins, including prostaglandin E2 and prostacyclin, which by activating EP2 and EP4, and IP receptors, respectively, expressed by nociceptive neurons causing their sensitization.[13–16] Nociceptive neuron sensitization is an increase in the excitability of nociceptors due to a facilitation to achieve the neuronal threshold to depolarization and therefore magnifies the response to noxious stimuli.[17] Indeed, the intraplantar (i.pl.) injection of KO_2 allows investigating inflammatory parameters such as edema and also the nociceptor neuron sensitization.[18] The intraperitoneal (i.p.) injection of KO_2 triggers abdominal contortions, a type of nociceptive behavior that is dependent on quick activation of ion channels that will induce neuronal activation. In fact, superoxide anion-triggered nociceptive neuron activation involves enhanced activity of various ion channels, including members of the transient receptor potential (TRP) family such as TRP vanilloid 1 (TRPV1). TRPV1 can be activated by temperatures above 43°C and acidic pH, and both TRP ankyrin 1 (TRPA1) and TRPV1 channels are involved in nociception induced by noxious chemical compounds.[19] The TRPA1 channel was particularly interesting for this study because it is responsible for the detection of ROS, pruritogens, mechanical hyperalgesia, and cold temperatures.[20] TRPA1 is mostly co-expressed with TRPV1 channels in dorsal root ganglia (DRG) neurons, which can interact with each other to facilitate their activity.[21–23]

KO_2 triggered inflammation and pain are also dependent on the induction of oxidative stress, including further superoxide anion production by the upregulation of $gp91^{phox}$, a heme-binding subunit of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase that mediates the final steps of electron transfer to molecular oxygen for superoxide ion generation.[18,24,25] Indeed, KO_2 triggered pain is amenable to treatment with apocynin (a NADPH oxidase inhibitor) and TEMPOL (a superoxide dismutase mimetic) along with evidence on the induction of superoxide anion production.[10]

trans-Chalcone (TC, 1,3-diphenyl-2-propen-1-one, Figure 1) is an open-chain flavonoid with anti-inflammatory properties in animal models of skin and hepatic inflammation[26–29] as well as in complete Freund's adjuvant inflammation and monosodium urate crystals in gouty arthritis.[30,31] Regarding the analgesic effects, TC reduced mechanical hyperalgesia in a gouty arthritis model,[31] thermal hyperalgesia induced by CFA,[30] abdominal contortions caused by acetic acid and phenyl-*p*-benzoquinone, and paw flinching/licking caused by formalin, CFA, capsaicin, and allyl isothiocyanate (AITC).[32] Interestingly, unlike most flavonoids, TC does not contain hydroxyl radicals or other chemical groups that

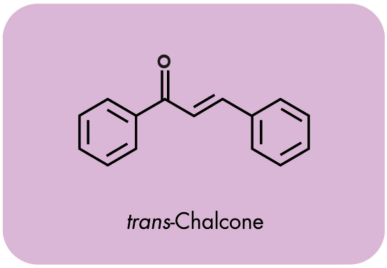

Figure 1 *Trans*-Chalcone *(TC – 1,3-diphenyl-2-propen-1-one) chemical structure*. Note that trans-chalcone does not present common chemical groups with antioxidant properties such as hydroxyl groups, making it different from most flavonoids or chalcones.[26]

would confer to it an antioxidant structural activity. In vitro systems without cells, thus, systems that do not allow induction of gene expression, but rather are designed to investigate the direct chemical antioxidant properties of a compound, TC is inactive. Therefore, the antioxidant properties of TC observed in vivo are not dependent on an inherent chemical antioxidant structural activity per se. Instead of directly inhibiting ROS similar to antioxidant molecules, TC seems to trigger cellular antioxidant responses against oxidative stress.[26]

Conceptually, understanding the biological role of TC can provide valuable insights into the mechanisms driving flavonoid activities that are independent of the intrinsic antioxidant properties of their chemical structure. The question is what are the biological activities and mechanisms of flavonoids that are independent of intrinsic structural chemical antioxidant properties? The singular chemical characteristic of TC among other flavonoids of lacking intrinsic antioxidant activity makes it a model molecule to answer this question. To our knowledge, the literature is missing investigating TC mechanisms in a model of inflammation and pain triggered by a ROS such as superoxide anion. Therefore, this study investigated the activity of TC in a KO_2 model and assessed its anti-inflammatory and analgesic mechanisms.

Materials and Methods

Animals and Ethical Approval

A total of 297 pathogen-free male Swiss mice, weighing 20–25 g, age 6–8 weeks, were provided by the central vivarium at Londrina State University and housed under 12 hours light/dark cycles at a climatized temperature (23 ± 1°C) with food and water *ad libitum*. For sample collection, mice were euthanized by terminal anesthetization with 5% isoflurane, followed by decapitation procedure as confirmation. All procedures were approved by the Animal Ethics Committee of Londrina State University (CEUA-UEL processes 24684.2016.72, December 8, 2016; and 050.2024, December 12, 2024). The methodology applied to this research was structured according to the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines and IASP (International Association for the Study of Pain) guidelines.

Writhing Response

The KO_2-induced writhing model was established as previously described[33] KO_2 (1 mg diluted in 250 µL of saline solution per animal) or saline solution (sterile 0.9% sodium chloride) was injected into the peritoneal cavities of mice 30 min after pre-treatment with TC (3, 10, or 30 mg/kg, p.o)., AMG-9810 (Santa Cruz Biotechnology, #sc-201477, 100 µM, 10 µL, intrathecal route), or HC-030031 (Santa Cruz Biotechnology, #sc-203994, 10 ng, 10 µL, intrathecal route). The intensity of overt pain-like behavior was expressed as the cumulative number of writhings over 20 min after the stimulus.

Mechanical Hyperalgesia and Edema

Mice that were randomly assigned for mechanical hyperalgesia assessment were placed in a wired surface to allow access to the plantar region of the inflamed paw. Upon acclimatization to the experimental room and apparatus, no signs of distress were observed. Mechanical hyperalgesia was assessed using the electronic version of von Frey filaments, which access the paw with a polypropylene tip and detects the force required for paw removal in grams. Measurements were repeated for 30 min and 1, 3, 5, and 7 h after the KO_2 injection. The results are expressed as the mean difference between the baseline and each time-point value (Δg) for the six animals. After establishing the baseline, edema measurements were obtained 1, 3, 5, and 7 h after KO_2 injection with the aid of a micrometer and are presented in millimeters (mm).

MPO Activity Assessment

MPO activity was used as an indirect measure of neutrophil and macrophage migration to stimulated paw tissue. Paw samples were collected 7 h after KO_2 stimulation in 200 µL of K_2HPO_4 buffer solution (pH 6.0) containing 0.5% HTAB and homogenized with a tissue homogenizer. After this step, the samples were centrifuged (14,000 x *g* at 4°C for 2 min), and the supernatant was separated. The supernatant of paw tissue homogenate was added to 200 µL of 50 mM phosphate buffer solution (pH 6.0) containing 0.167 mg/mL o-dianisidine dihydrochloride and 0.015% hydrogen peroxide. Absorbance was recorded at 450 nm using a microplate spectrophotometer (Multiskan GO, Thermo Fischer

Scientific). MPO activity was expressed as MPO activity (number of neutrophils x10^4 / mg of tissue) compared with a neutrophil standard curve.[26]

Total Antioxidant Capacity

The 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS, Sigma, #1888) and ferric reducing antioxidant power (FRAP) assays were performed to quantify the ability of the samples to resist oxidative damage. The paw tissue samples were homogenized in ice-cold phosphate-buffered saline (PBS) containing KCl 1.15% and centrifuged at 200 × *g* at 4°C for 10 min. The supernatants were transferred to new microtubes for the assays. On 96-well plates, ABTS reagent was added to the supernatant or standards for the calibration curve, allowed to incubate for 6 min at room temperature, and the absorbance of the samples was read in a microplate spectrophotometer at 730 nm. To evaluate FRAP levels, 150 µL of FRAP reagent was added to the supernatant or standards for the calibration curve and read at 595 nm (Multiskan GO, Thermo Fischer Scientific). The results were compared with the respective Trolox standard curves and expressed as Trolox equivalent/mg of tissue for both evaluations.[34,35]

Lipid Peroxidation and Superoxide Production

The thiobarbituric acid-reactive substance (TBARS) test is an indicator of lipid peroxidation. The test detects the presence of malondialdehyde (MDA) in samples because it reacts with 2-thiobarbituric acid (TBA, 1%, MP Biomedicals, #02190284) that in the presence of Iron(III) chloride solution 1 mM($FeCl_3$, Vetec, #342), ascorbic acid 1 mM (Amresco, #0143), 50% trichloroacetic acid (TCA – Sigma Aldrich, #T6399) at 95°C produces a color change to pink proportionally to the level of MDA-TBA complex, a measure of lipid peroxidation. Absorbance was measured at 572 and 535 nm, and the results were expressed as 572–575 nm OD/mg of protein.[36] Superoxide anion production was determined using the nitroblue tetrazolium (NBT, Roche, #11585029001) test as previously described adapted to microplates.[27,35] Briefly, sample homogenates were incubated in microplates with 1 mg/mL NBT reagent for 1 hour at room temperature and protected from light. After incubation, the supernatant was removed, and 2 M KOH solution and pure dimethyl sulfoxide (DMSO) were added to the microplates. Relative production of superoxide anions was measured spectrophotometrically at 600 nm (Multiskan GO; Thermo Fisher Scientific). The results are expressed as OD/mg of protein.

Cytokine Levels Assessment

Cytokine levels were assessed using ELISA in paw samples collected 3 h after the KO_2 stimulus. Samples were collected in phosphate-buffered saline, homogenized, and centrifuged (400 x *g* at 4°C for 15 min), and the supernatant was used to measure the levels of IL-1β, TNF-α, IL-10, IL-6, and IL-33. Assays were conducted according to the manufacturer's instructions (eBioscience; IL-1β #88-7013; TNF-α #88–7328 o #88–7324; IL-10 #88-7804; IL-6 #88-7064, and IL-33 #88-7333). Briefly, 96-well plates were sensitized with their respective capture antibodies 1 day before the experiments. In the following day, the wells were washed (0,05% Tween-20 PBS), blocked for 1 h, washed again, standard and samples were added to their respective wells and incubated in a state of slow orbital agitation for 2 h at room temperature before carrying out the final washing, detection antibody incubation for 2 h, followed by washing, substrate incubation and stop solution addition steps. Spectrophotometer-readings were performed at 450 nm and 570 nm (Multiskan GO; Thermo Fisher Scientific). The values of 570 nm were subtracted from those of 450 nm and data were analyzed. The results were expressed as picograms (pg) of each cytokine per 100 mg of tissue.

Ca^{2+} Influx Imaging

The influx of calcium ions in afferent neurons is an indicator of their activity, enabling comparison of influx thresholds between experimental groups. Mice were pre-treated with TC, and the right hind paw was stimulated 30 min later. After 5 h, the mice were terminally anesthetized, peripheral blood was manually removed after decapitation, and the right DRG (L4, L5, and L6) was harvested in Dulbecco's modified Eagle's medium (DMEM) high glucose supplemented with 10% fetal bovine serum (FBS). DRG were enzymatically disrupted with collagenase A/dispase II solution supplemented with 5 mM $CaCl_2$ (Collagenase A, Roche, #10103578001, Dispase II, Roche, #04942078001) and incubated at 40°C until

DGR opened fibers were visualized, followed by pipetting until complete dissociation. The cells were then centrifuged (200 x g, for 5 min) to obtain a monolayer of DRG axotomized neurons that were placed over a glass-bottom culture plate pre-coated with laminin (1.2 mg/mL, Gibco, #23017015). Cells were kept at 37°C, 5% CO_2 overnight in neurobasal culture medium (NBM, Gibco, #21103049) supplemented with 2% B27 serum-free supplement (Gibco, #17504044) 1% Pen/Strep (Cellgro, #15140-163), 1% L-glutamine (Gibco, #25030164), 0.004% cytabarine (ARA-C, Merck, #C3350000), 0.004% glial-derived neurotrophic factor (GDNF, Thermo Fischer Scientific, #450-02-50UG), and 0.002% nerve growth factor (NGF, Scientific, #450-34-5UG). Four culture plates were seeded for each experimental group, composed of six mice, and kept overnight at 37°C, 5% CO_2 for stabilization. The culture plates were washed, and cell adherence was confirmed. The experimental sets were divided into one plate per group (Saline, KO_2 stimulus after vehicle treatment, and KO_2 stimulus after TC treatment). Cells were then loaded with 1.2 μM Fluo-4-AM probe (Invitrogen, #F14217) for 40 min, the plates were washed, and the medium was replaced with balanced salt solution (HBSS, Gibco, #14170138) with calcium and magnesium for neuronal stimuli imaging using a confocal microscope (TCS SP8, Leica Microsystems). To assess TRPV1 and TRPA1 activation, DRG plates were recorded for 4 min, divided into 1 min of initial reading (0-s mark, baseline values), followed by stimulation with capsaicin (1 μM, TRPV1 agonist, Sigma-Aldrich, #M2028) or Allyl isothiocyanate (AITC, 100 nM, TRPA1 agonist, Sigma-Aldrich, #377430) for 2 min at the 60s mark and KCl (40 mM, a viability control for cells) for 1 min at the 180s mark. Calcium influx was analyzed based on the mean fluorescence intensity detected in each neuron using the LAS X Software over time (Leica Microsystems). The Fluo-4 probe is detected in the green fluorescence range (Ex/Em of Ca^{2+}–bound form 494/506 nm). However, the signal intensity is converted by the confocal microscope software into a color gradient in which blue/purple represents low fluorescence intensity and red is the maximum fluorescence intensity. Therefore, minimum levels of calcium are observed as blue neurons and maximum increase of calcium influx is observed as red neurons. This software pseudo-color conversion allows an easier visual interpretation of the results. Nonetheless, it is important to mention that the fluorescence intensity is calculated by the LAS X Software. Experimental recordings were obtained using a 20x objective lens.[37]

RT-qPCR

Paw samples were collected in lysis reagent (QIAzol, QIAGEN, #79306), snap-frozen in liquid nitrogen, and stored at −80°C. Total RNA was extracted according to the manufacture's instructions. Nucleic acid purity and integrity were assessed by spectrophotometry, adopting 1.6–2 cut off for Reading the 260/230 nm and 2.0 ± 0.1 for 260/280 nm ratios, respectively. The QuantiTect Reverse Transcription kit (QIAGEN, #205314) was used to obtain cDNA, and the QuantiNova SYBR Green (QIAGEN, #208056) kit was used for quantitative PCR analysis. Gene expression was determined using the comparative $2^{-(\Delta\Delta Ct)}$ method after *Actin* housekeeping gene correction. The primer sequences used are listed in Table 1.

Fluorescent Western Blot

Western blotting was performed according to Pinto et al (2025).[38] Briefly, paw samples were collected in a lysis buffer (RIPA, Thermo Scientific, 89901) containing protease (cOmplete™ ULTRA Tablets, Roche, #5892970001) and phosphatase inhibitors (PhosSTOP, Roche, #4906845001). After mechanical dissociation, the samples were centrifuged for 20 min at 16,000 × g and the supernatant was transferred to a new tube for total protein quantitation. The samples were equalized, subjected to vertical electrophoresis, and transferred to nitrocellulose membranes by wet transfer. Transfer

Table 1 Primer Sequences

	Sense	Antisense
gp91phox (*Cybb* gene)	5′ AGC TAT GAG GTG GTG ATG TTA GTG G 3′	5′ CAC AAT ATT TGT ACC AGA CAG ACT TGA G 3′
β-actin (*Actin* gene)	5′ AGC TGC GTT TTA CAC CCT TT 3′	5′ AAG CCA TGC CAA TGT TGT CT 3′
Nrf2 (*Nfe2l2* gene)	5′ TCA CAC GAG ATG AGC TTA GGG CAA 3′	5′ TAC AGT TCT GGG CGG CGA CTT TAT 3′
COX-2 (*Cox2* gene)	5′ GTG GAA AAA CCT CGT CCA GA 3′	5′ GCT CGG CTT CCA GTA TTG AG 3′

quality was confirmed using Ponceau red and the membranes were blocked with 5% bovine serum albumin (BSA, Sigma-Aldrich, #A9418). The primary antibodies p-NF-κB p65 mouse monoclonal (1:200, 27. Ser 536, Santa Cruz Biotechnology, #sc-136548,) and β-actin rabbit monoclonal (1:1000, D6A8, Cell Signaling, #8457), and secondary antibodies goat anti-mouse IgG Alexa Fluor™ 488 (1:200, Invitrogen, #A11001) and goat anti-rabbit IgG Alexa Fluor™ 790 (1:200, Invitrogen, #A11367), diluted in 3% BSA Tris-buffer saline (TBS), were selected for this study. The primary antibodies were incubated overnight with slow agitation. Membranes were then washed three times with TBS for 10 min and incubated with fluorescent secondary antibodies for 1 hour. Membranes were washed repeatedly, photo-documented (ImageQuant 800 – Cytiva), and fluorescence intensity was quantitated applying the software Image Lab 6.1 – Bio-Rad. Relative expressions were calculated based on the β-actin fluorescence intensity.

Experimental Design

TC (*trans*-chalcone, Santa Cruz Biotechnology, #sc-204681) was diluted in saline solution with 20% Tween 80, and treatments were administered orally (per oral, P.O.) by intra-gastric gavage 30 minutes prior to KO_2 (Potassium Superoxide, Sigma-Aldrich, #278904) injection. The TC dose was based on a prior study of our group that performed dose–response curves.[31] The stimulus with KO_2 was performed by intraperitoneal (i.p.) or intraplantar (i.pl., subcutaneously into the plantar surface of the right hind paw) injection, not both. The i.p. administration of KO_2 was used to induce abdominal contortions and was used solely in this experiment. The i.pl. administration was used to induce mechanical hyperalgesia and paw edema as well as for collecting samples for all other measurements described below. It is important to reinforce the information that animals received KO_2 by i.p. or i.pl. route, no animal received both stimuli. TC analgesic effect was assessed based on overt pain-like behavior in which the i.p. administration was used to observe the number of abdominal contortions displayed due to the elevation of superoxide anion. Therefore, TC treatment (30 mg/kg) was administered 30 min before abdominal contortion induction by i.p. injection of 1 mg of KO_2 per animal (total of 18 mice – 6 mice per group). This assay was repeated in another set of animals to assess the effect of intrathecal (i.t.) administration of AMG-9810 (a TRPV1 antagonist) (total of 18 mice – 6 mice per group) or HC-030031 (a TRPA1 antagonist) (total of 18 mice – 6 mice per group) against KO_2 i.p. injection triggered abdominal contortions. Abdominal contortions were assessed over 20 min after KO_2 i.p. administration. For all the following experiments, KO_2 (30 μg diluted in 25 μL saline solution per animal) was injected i.pl. 30 min after treatment with TC. Due to KO_2 fast inactivation,[18] the stimulus was always injected–30–180 seconds after saline dilution to enable consistent results. Mechanical hyperalgesia was assessed using electronic von Frey 0.5, 1, 3, 5, and 7 h after the KO_2 stimulus, and paw edema was measured 1, 3, 5, and 7 h after KO_2 injection. The same animals were used to assess mechanical hyperalgesia and paw edema (total of 18 mice – 6 mice per group). Leukocyte recruitment to the plantar tissue was evaluated by MPO activity assessed 7 hours after KO_2 paw injection (total of 15 mice – 5 mice per group). A total of 15 mice (5 mice per group) were used to determine superoxide anion production (NBT). The same animals were used to evaluate lipid peroxidation levels (TBARS), and antioxidant capacity (FRAP and ABTS assays) in paw tissue samples collected 3 hours after KO_2 injection (total of 18 mice – 6 mice per group). Paw skin samples were also collected 3 hours after KO_2-stimilus for RT-qPCR (total of 15 mice – 5 mice per group) or ELISA (two sets of 15 mice – 5 per group – were used because samples were not sufficient for all ELISA dosage. One set to assess IL-1β, TNF-α, and IL-33 levels; and the other to investigate IL-10 and IL-6 levels), and Western Blot assays (total of 12 mice – 4 mice per group). Calcium imaging using confocal microscopy of cultured DRG-derived neurons collected 5 h after KO_2 i.pl. injection was used to evaluate the activity of the $TRPV1^+$ and $TRPA1^+$ DRG nociceptive neurons. Each group presented 4 plates, and each plate was a pool of the DRG of 5 mice (total of 60 mice divided in 20 mice per group; 4 pools of 5 mice per group). The neuronal fluorescence was calculated individually by circulating/selecting each cell and annotating the values provided by the confocal microscope software. The range of live cells (KCl responsive at the end of the experiment) that were responsive to capsaicin (TRPV1+) or AITC (TRPA1+) ranged between 174 and 352 neurons with a mean ± s.e.m. of 283.5 ± 42.16. The total number of mice was 297.

Statistical Analysis

The sample size was estimated using the G*Power 3.1. software[39] based on previous studies.[11,18,24] GraphPad Prism software (version 9.0) was used for statistical analysis and graphical representation. Data indicates the number of

biological replicates (n), which is the number of mice or the number of pools of mice samples in each group. To obtain the values of biological replicates, technical replicates were performed. For the technical replicates for TC activity in the abdominal contortions test, 3 independent experiments counted the number of responses with n = 6 biological samples per group. For mechanical hyperalgesia and edema, 3 technical replicate measurements were performed at each time point in every animal with n = 6 biological samples per group. For Western blot we used 4 mice per group without replication to allow assessment of band intensity in the same membrane for all groups. The method of block randomization was used to randomize subjects into groups which result in equal sample sizes at all time points for each assay. We applied a fluorescent WB to allow determining the intensity of housekeeping control also in the same membrane as the target protein as well as presenting the image of both proteins together. For all other ex-vivo quantitation assays, all dosages were performed in duplicate technical replicates with an n = 5 biological replicates per group (MPO, NBT, cytokines, and RT-qPCR) or n = 6 biological replicates per group (FRAP, ABTS, and TBARS). In the abdominal contortions plus treatment with AMG-9810 or HC-030031, 3 independent experimenters counted the number of responses, and a total of 6 biological replicates per group was used. In the calcium assay, the ipsilateral L4-L6 DRG of 5 mice per group were collected to form 1 pool/plate representing 1 biological sample. A total of 4 biological samples were used for each group in the calcium assay. The total number of neurons analyzed per plate ranged from 174 to 352 with a mean ± s.e.m. of 283.5 ± 42.16. The neurons were selected first by their viability at the end of the assay, which was the response to KCl, and then by the response to capsaicin or AITC to identify $TRPV1^+$ and $TRPA1^+$ neurons, respectively. Pre-tests Shapiro–Wilk and Brown-Forsythe tests were used to verify the normality and equality of the group variances, respectively. To analyze multiple time points for normally distributed data, two-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test was performed. One-way ANOVA followed by Tukey's test was used for normal column analysis. Welch's ANOVA test followed by Dunnett's T3 multiple comparisons test was applied when data presented normal distribution, but unequal variances. Non-parametric data were evaluated using the Kruskal–Wallis' test followed by Dunn's multiple comparison test. The groups were considered statistically different if the p-value was ≤0.05.

Results

TC Inhibits Overt Pain-Like Behavior, Mechanical Hyperalgesia, Edema, and Neutrophil/ Macrophage Recruitment Induced by Superoxide Anion

This study aimed to determine whether TC can reduce the pain phenotype induced by a superoxide anion donor, KO_2, and its mechanisms. The dose of 30 mg/kg of TC was selected based on a prior study of our group using a gout arthritis model and investigating the activity of TC.[31] Overt pain-like behavior was quantified by the number of abdominal contortions induced by i.p. injection of KO_2 after treatment with TC (30 mg/kg) or vehicle. TC treatment reduced the writhing response (Figure 2A) by 74.6%. In Figure 2A, KO_2 was injected i.p. because only stimuli that are injected in the abdominal cavity trigger the overt pain-like behavior of writhing response. In the following experiments, KO_2 was injected by i.pl. route to investigate hyperalgesia, inflammation, production of mediators, intracellular signaling pathways, and neuronal activation.

The results indicated that KO_2 rapidly induced mechanical hyperalgesia, observed 30 min after stimulus injection, and the response was sustained until at least the 7th hour (Figure 2B). Treatment with TC reduced mechanical hyperalgesia at all time-points analyzed (30 min, 56.1%; 1 h, 68.2%; 3 h, 70%; 5 h, 83.2%; and 7 h, 90.2% inhibition), further indicating the inhibition of nociception. Similarly, the flavonoid significantly reduced the extent of paw edema (Figure 2C), which was observed from the 1st (74.5%) to the 3rd hour (52.5%) after KO_2 injection. As shown in Figure 2D, TC treatment inhibited KO_2-induced neutrophil/macrophage recruitment 7 h after stimulation, reducing MPO activity by 46.7%.

It is important to mention that KO_2 triggered inflammation and pain are amenable by the opioid morphine, which reinforces that nociceptive behaviors are under investigation. Quercetin, which has the main structural chemical groups to explain the direct antioxidant effects a flavonoid, also inhibits inflammation and pain caused by KO_2, demonstrating the role of oxidative stress in the model.[18] TEMPOL, a superoxide anion dismutase mimetic[40] as well as the NADPH oxidase inhibitor, apocynin,[10] inhibit the inflammation and pain caused by KO_2, further supporting that in addition to the

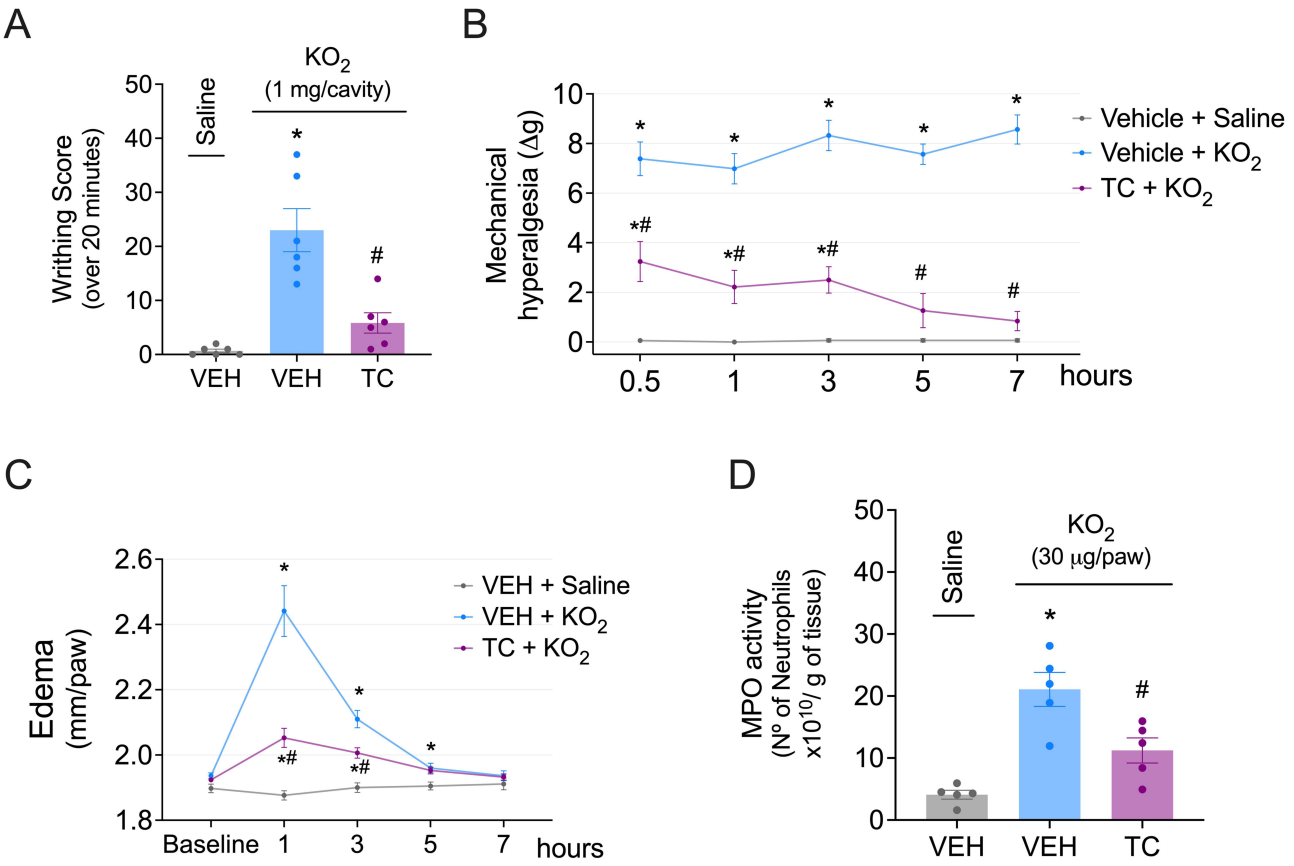

Figure 2 *TC inhibits writhing response, mechanical hyperalgesia, edema and neutrophil/ macrophage recruitment caused by KO_2 stimulus.* (**A**) Writhing response was induced by intraperitoneal (i.p.) administration of KO_2 (1 mg/cavity) 30 min after TC treatment (30 mg/kg) and assessed for 20 minutes – n = 6. Statistical analyses were performed by Welch's ANOVA test followed by Dunnett's T3 multiple comparisons test. (**B**) Mechanical hyperalgesia was measured by electronic von Frey 0.5, 1, 3, 5 and 7 hours after KO_2 i.pl. injection. Statistical analyses were performed by Two-way ANOVA followed by Tukey's multiple comparisons test – n = 6. (**C**) Paw edema was measured after 1, 3, 5 and 7 hours from KO_2 stimulus. Statistical analyses were performed by Two-way ANOVA followed by Tukey's multiple comparisons test – n = 6. (**D**) MPO activity in the injected paw was measured 7 hours after stimulus – n = 5. Statistical analyses were performed by One-way ANOVA followed by Tukey's multiple comparisons test. Results are presented as means ± standard error mean (SEM). *p-value ≤ 0.05 vs. vehicle + saline group; #p-value ≤ 0.05 vs. vehicle + KO_2 group.

initial release of superoxide anion, KO_2 triggers further production of ROS in the model. Celecoxib, which is a selective cyclooxygenase-2 (COX-2) inhibitor also reduces KO_2 inflammation and pain as well as KO_2 triggers *Cox-2* mRNA expression.[18] These prior data on the KO_2 model suggest that the investigation of the anti-inflammatory and analgesic mechanisms of TC should start around the known pathological mechanisms triggered by superoxide anion.

TC Induces Antioxidant Response After KO_2 Injection with Upregulation of the Transcription Factor Nrf2 (Nuclear Factor Erythroid 2-Related Factor 2) mRNA Expression

Considering that KO_2 is a donor of the superoxide anion, the next step after confirming the biological relevance of TC as a treatment against inflammation and pain was to determine whether TC could induce antioxidant responses to counteract KO_2 oxidative stress. FRAP and ABTS assays were performed. While KO_2 reduced the antioxidant capacity in the paw skin, TC was able to upregulate antioxidant levels, increasing ferric reducing power by 69.6% (Figure 3A) and elevating the scavenge of ABTS radical cation by 42.9% (Figure 3B). Moreover, Nrf2 mRNA expression was upregulated by 109% (Figure 3C) in mice that received TC treatment. It is possible that TC triggers endogenous antioxidants genes expression up-regulation via Nrf2 to increase antioxidant responses, because TC is not an antioxidant per se.[26]

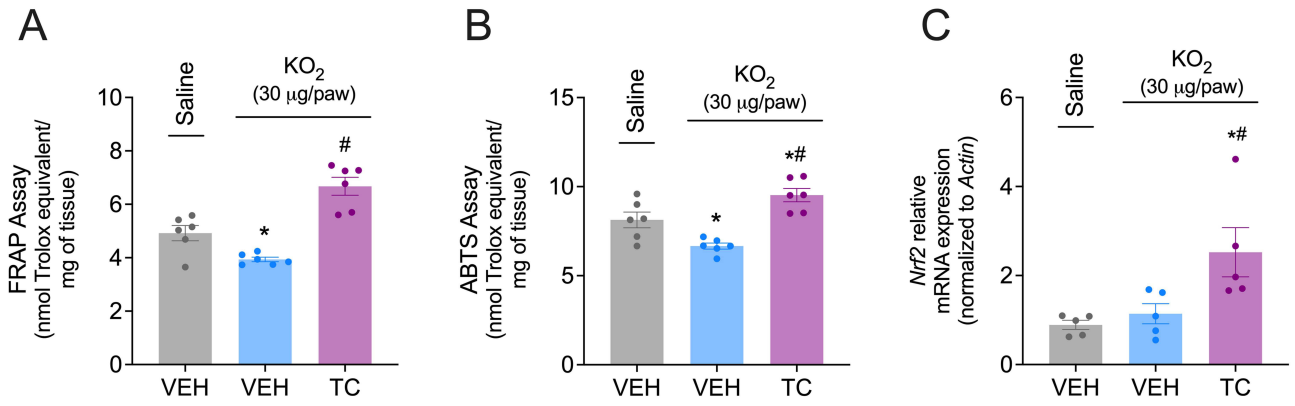

Figure 3 *TC induces antioxidant response against KO_2 triggered inflammation in mice.* (**A**) Paw skin FRAP (n = 6), (**B**) ABTS (n = 6), and (**C**) Nrf2 mRNA expression by RT-qPCR (n = 5) were assessed. Statistical analyses were performed by One-way ANOVA followed by Tukey's multiple comparisons test. Results are presented as means ± SEM. *p-value ≤ 0.05 vs. vehicle + saline group; #p-value ≤ 0.05 vs. vehicle + KO_2 group.

TC Reduces KO_2-Triggered Oxidative Stress by Targeting NADPH Oxidase

Regarding the flavonoid effects on paw tissue oxidative stress indicators triggered by KO_2 stimulus, lipid peroxidation was inhibited by 25.9% (Figure 4A) and superoxide anion levels were reduced by 49.9% (Figure 4B) upon TC pre-treatment. These effects on reducing oxidative stress might be explained, at least in part, by the downregulation *Cybb* expression, the gene that encodes $gp91^{phox}$, one of the components of the NADPH oxidase complex, which generates superoxide anions. *Cybb* (Figure 4C) expression was reduced by 88.6% in the group that received TC pre-treatment.

TC Inhibits Inflammatory Cytokine Production Triggered by KO_2

Cytokines such as IL-1β, TNF-α, IL-10, IL-6, and IL-33 have been demonstrated to mediate[10,11] or be modulated by drugs that cause analgesia and reduce inflammation in the KO_2 model.[7–9,24,41] Therefore, we investigated whether TC would alter their production. Our results demonstrated an upregulation in all analyzed cytokine levels (Figure 5A-E) triggered by KO_2, whereas treatment with TC reduced the protein levels of IL-1β (35.3%; Figure 5A), TNF-α (37%; Figure 5B), IL-10 (41.4%; Figure 5C), IL-6 (52.2%; Figure 5D), and IL-33 (28.3%; Figure 5E).

TC Inhibits the Activation of NF-κB and COX-2 mRNA Expression Induced by KO_2

The effects of TC on inflammation triggered by KO_2 have also been observed in activation of the NF-κB pathway. The detection of the activated form of NF-κB p65 (phosphorylated-NFκβ p65) was inhibited by 42.5% (Figure 6A and B) in

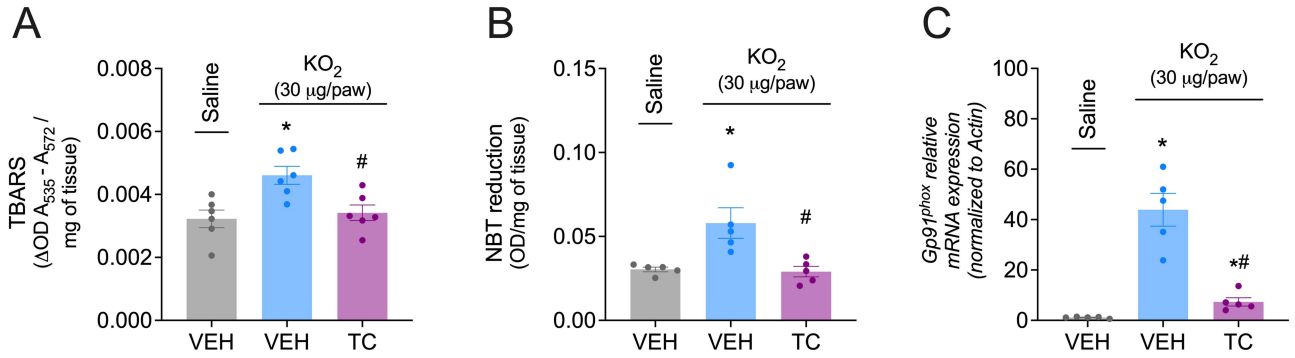

Figure 4 *TC reduces oxidative stress triggered by KO_2 in mice.* (**A**) Paw skin TBARS (n = 6) (**B**), NBT (n = 5), and (**C**) $gp91^{phox}$ mRNA expression (*Cybb* gene) (n=5) were assessed. For TBARS and NBT, Statistical analyses were performed by One-way ANOVA followed by Tukey's multiple comparisons test. To analyze $gp91^{phox}$ mRNA expression, statistical analyses were performed with Welch's ANOVA test followed by Dunnett's T3 multiple comparisons test. Results are presented as means ± SEM. *p-value ≤ 0.05 vs. vehicle + saline group; #p-value ≤ 0.05 vs. vehicle + KO_2 group.

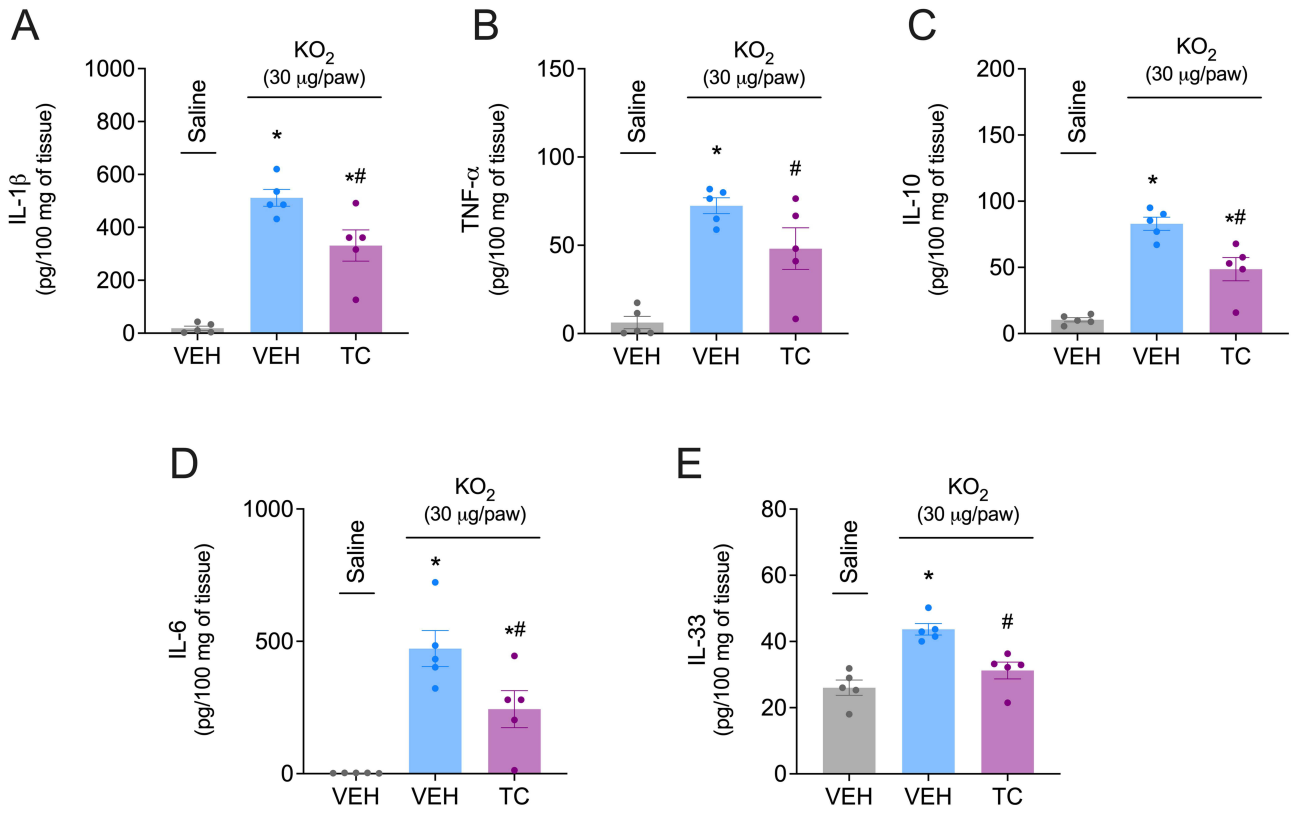

Figure 5 *TC inhibits KO_2-triggered cytokine production.* Production of (**A**) IL-1β – n = 5, (**B**) TNF-α – n = 5, (**C**) IL-10 – n = 5, (**D**) IL-6 – n = 5, and (**E**) IL-33 – n = 5 were quantified by ELISA. Statistical analyses were performed by One-way ANOVA followed by Tukey's multiple comparisons test. Results are presented as means ± SEM. *p-value ≤ 0.05 vs. vehicle + saline group; #p-value ≤ 0.05 vs. vehicle + KO_2 group.

the group that received TC pre-treatment. Furthermore, TC treatment also inhibited COX-2 (*Cox2* gene) (Figure 6C) mRNA expression triggered by the superoxide donor in 66.2%.

Under pro-inflammatory conditions, active (phosphorylated) NF-κβ translocates into the nucleus and induces the expression of inflammatory molecules, including cytokines and enzymes such as COX-2. Evidence supports that KO_2 triggers NF-κB activation and the pharmacological inhibition of the activation of this transcription factor reduces inflammation and pain by reducing cytokine production and *Cox*-2 mRNA expression.[7–9] Therefore, TC-mediated inhibition of NF-κB activation is responsible, at least in part, for the reduction in cytokine production levels (Figure 5) and *Cox-2* mRNA expression (current Figure 5C).

TC Inhibits the Sensitization of $TRPV1^+$ Nociceptive Neurons

Oxidative stress and NF-κB-dependent production of cytokines and COX-2 derived prostanoids are the target mechanisms that explain the anti-inflammatory and analgesic activities of TC. Nevertheless, evidence supports that superoxide anion and other ROS activate TRPV1+ and TRPA1+ neurons to induce pain.[4] Therefore, we further investigated the analgesic mechanisms of TC using a calcium assay to determine DRG $TRPV1^+$ and $TRPA1^+$ nociceptive neuron activity. First, we observed that the KO_2 writhing response was inhibited by an antagonist of TRPV1 (AMG-9810), demonstrating an endogenous role of TRPV1 in the overt pain-like behavior triggered by KO_2 (Figure 7A).

Next, in a different set of experiments, calcium influx was assessed ex vivo, the DRG nociceptive neurons of mice that received TC or vehicle pre-treatment and were stimulated with KO_2 or saline i.pl. injections were collected, processed, and subjected to in vitro calcium influx. The neuronal population was determined by responsiveness to capsaicin ($TRPV1^+$). KO_2 stimulation induced DRG-neuron sensitization, as observed by a baseline increase of 41% in the fluorescence intensity of cultured neurons, as observed in the representative culture images (Figure 7B), Venn diagram (Figure 7C), calcium tracer (Figure 7D), and bar panel (Figure 7E). The TC pre-treated group demonstrated

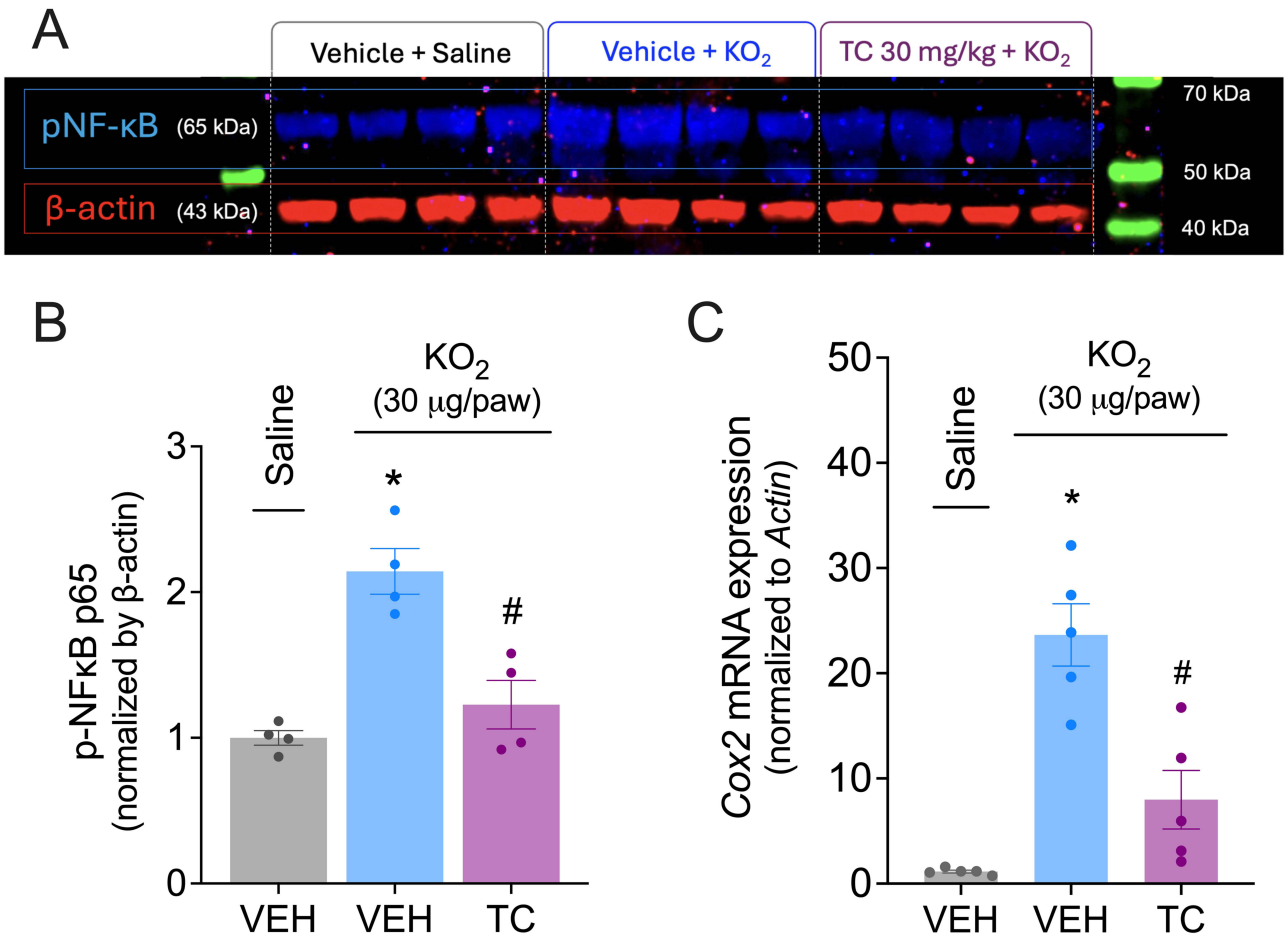

Figure 6 *TC inhibits NF-κB activation and Cox2 mRNA expression induced by KO_2.* (**A** and **B**) p-NF-κB (blue) and β-actin (red) were assessed by Fluorescent Western Blot, which allowed dual detection in the same membrane – n = 4. (**C**) mRNA expression of *Cox2* was measured via RT-qPCR – n = 5. Statistical analyses were performed by One-way ANOVA followed by Tukey's multiple comparisons test. Results are presented as means ± SEM. *p-value ≤ 0.05 vs. vehicle + saline group; #p-value ≤ 0.05 vs. vehicle + KO_2 group.

reduced baseline activation (26%) compared with the vehicle treated group that also received KO_2 i.pl. stimuli (Figure 7E). We also observed that TC pre-treatment reduced the calcium influx in $TRPV1^+$ neurons, as indicated by a 36% reduction in fluorescence intensity upon capsaicin-induced ionic channel activation (Figure 7E), as well as a 50.5% reduction in the number of activated neurons (Figure 7C).

TC Inhibits the Sensitization of $TRPA1^+$ Nociceptive Neurons

First, we observed that HC-030031 (a TRPA1 receptor antagonist) inhibited KO_2 triggered writhing response, demonstrating that TRPA1 presents an endogenous role in the overt pain-like behavior of abdominal contortions in the KO_2 model (Figure 8A).

Afterwards, in a different set of experiments, the activation of $TRPA1^+$ neurons was assessed by the calcium levels. For the calcium protocol, mice were treated with vehicle or TC and then received the i.pl. stimulation with KO_2. The DRG of these mice that underwent the in vivo treatment/stimulation protocol were collected for calcium assay. The DRG neurons of mice that received vehicle plus KO_2 i.pl. stimulation presented higher intracellular calcium levels compared to the mice that received TC plus KO_2 and the saline group at baseline, as can be observed in the representative culture images (Figure 8B), Venn diagram (Figure 8C), calcium trace (Figure 8D), and bar panel quantitation (Figure 8E) confirm that the i.pl. injection of KO_2 induces the sensitization of DRG neurons. Indeed, upon addition of the TRPA1 agonist AITC to the cell culture media, 73.4% of viable neurons (KCl responsive at the end of the assay) showed an

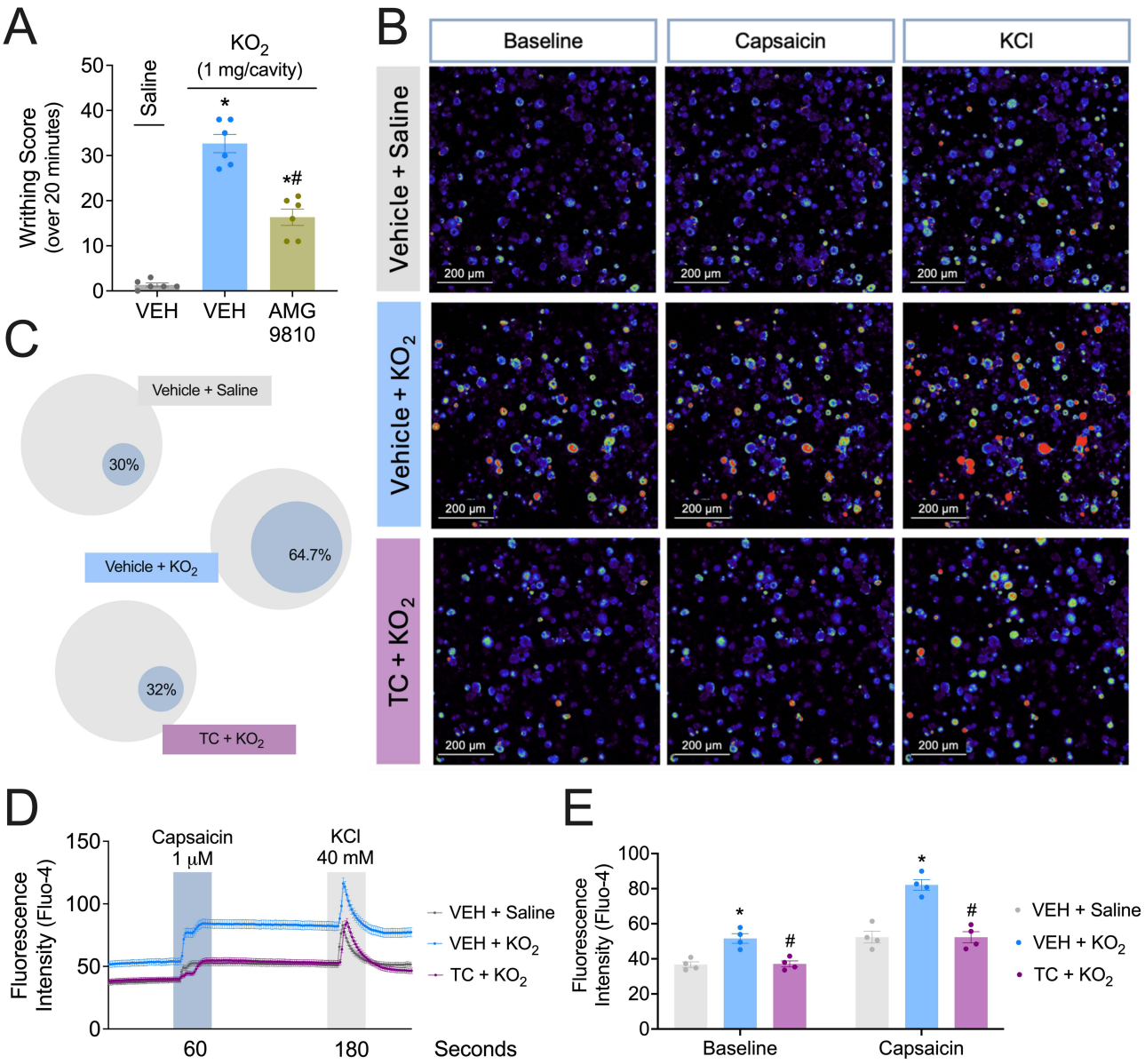

Figure 7 *KO_2 writhing response is TRPV1 dependent and TC inhibits KO_2-induced TRPV1+ nociceptor activation*. (**A**) Effect of intrathecal (i.t.) pre-treatment with the TRPV1 antagonist, AMG-9810 (100 µM, 10 µL), or its vehicle against the writhing response triggered by KO_2 (1 mg/animal, i.p.) – n = 6. Statistical analyses were performed by the Welch's ANOVA test followed by Dunnett's T3 multiple comparisons test. To assess the effects of TC on $TRPV1^+$ neuron sensitization after KO_2 i.pl. injection, DRG neurons from the injected paw side of L4, L5 and L6 were collected 5 hours after stimulus and processed. Plates with Fluo-4-labeled cells were recorded for 4 min: 1 min of initial reading, followed by stimulus with capsaicin (TRPV1 agonist, 1 µM in HBSS) for 2 min at the 60s-mark, and with KCl for 1 min at the 180s-mark (40 mM, activates all neurons). (**B**) Representative pictures throughout the 4 min of data acquired at baseline, capsaicin stimulus, and KCl activation, for vehicle + saline, vehicle + KO_2, and TC + KO_2 groups. (**C**) Venn's diagram indicating the percentage of DRG-derived neurons activated after capsaicin stimulus. (**D**) Fluorescence intensity tracers throughout the 4 min of recording. (**E**) Comparative mean fluorescence intensity between vehicle + saline, vehicle + KO_2, and TC + KO_2 groups at baseline and capsaicin-induction timepoints. Results are means ± SEM of four culture dishes per group and each culture dish was a pool of DRG from 5 mice (n=4 culture dishes). Statistical analyses were performed by the two-way ANOVA followed by Tukey's multiple comparisons test (**E**). *p-value ≤ 0.05 vs. vehicle + saline group; #p-value ≤ 0.05 vs. vehicle + KO_2 group.

increase in calcium influx in the vehicle plus KO_2 group, whereas the saline group (52.3%) and TC plus KO_2 group (48.7%) showed calcium influx reaching nearly half of the neurons (Figure 8C). TC pre-treatment inhibition of KO_2-triggered nociceptive neuron sensitization was also observed by fluorescence intensity, which was 37.5% lower in the TC plus KO_2 group than in the vehicle plus KO_2 group upon AITC stimulation (Figure 8E).

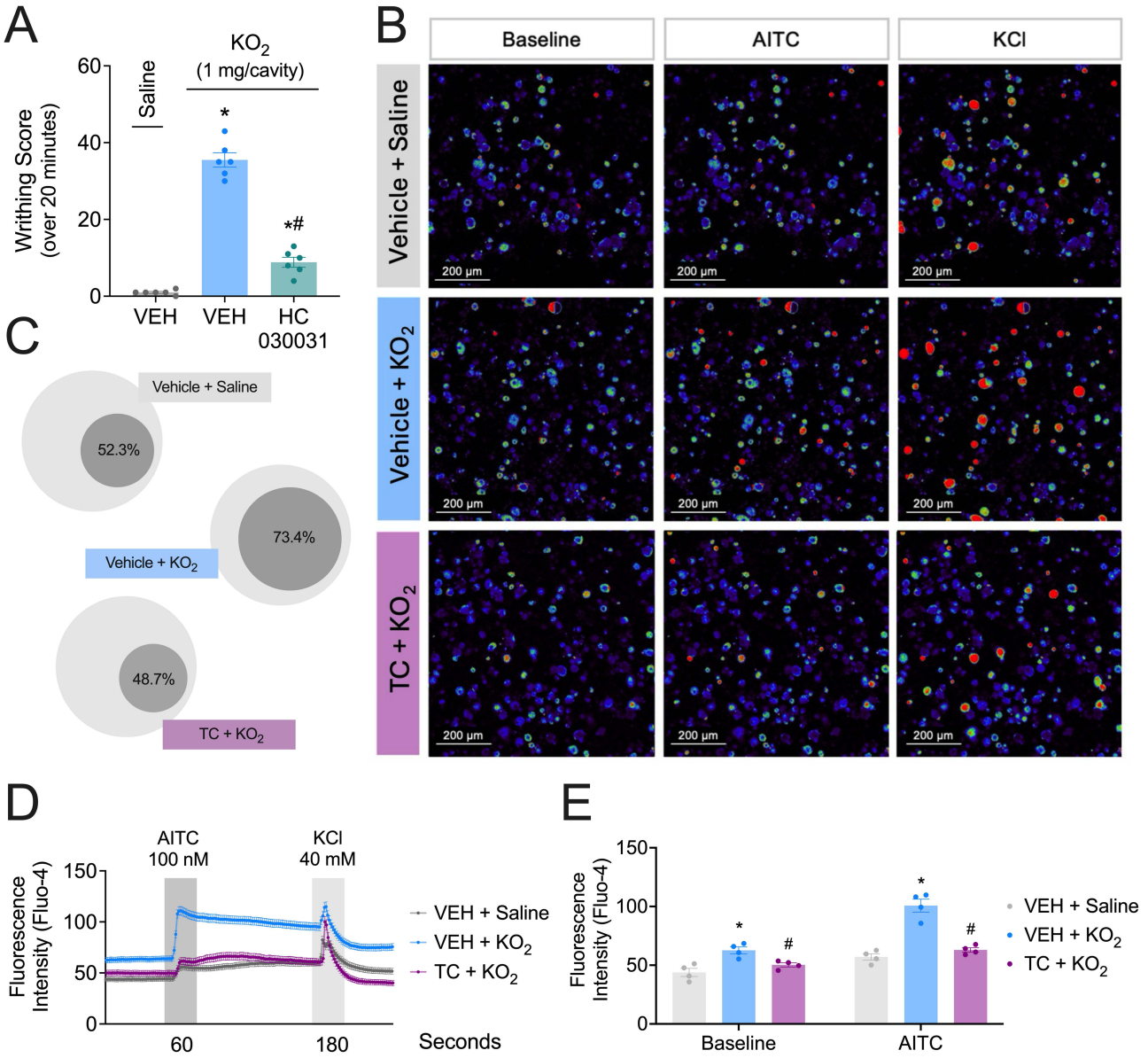

Figure 8 *KO_2 writhing response is TRPA1 dependent and TC inhibits KO_2-induced TRPA1$^+$ nociceptive neuron activation.* (**A**) Effect of i.t. pre-treatment with the TRPA1 antagonist, HC-030031 (10 ng, 10 µL), or its vehicle against the writhing response triggered by KO_2 (1 mg/animal, i.p.) – n = 6. Statistical analyses were performed by one-way ANOVA followed by Tukey's test (A). To assess the effects of TC on TRPA1$^+$ neuron sensitization after KO_2 i.pl. injection, DRG neurons from the right side of L4, L5 and L6 were collected 5 hours after stimulus and processed. Plates with Fluo-4-labeled cells were recorded for 4min: 1min of initial reading, followed by stimulus with AITC (TRPA1 agonist, 100 nM in HBSS) for 2 min at the 60s-mark, and with KCl for 1 min at the 180s-mark (40 mM, activates all neurons). (**B**) Representative pictures throughout the 4 min of data acquired at baseline, AITC stimulus, and KCl activation, for vehicle + saline, vehicle + KO_2, and TC + KO_2 groups. (**C**) Venn's diagram indicating the percentage of DRG-derived neurons activated after capsaicin stimulus. (**D**) Fluorescence intensity tracers throughout the 4 min of recording. (**E**) Comparative mean fluorescence intensity between vehicle + saline, vehicle + KO_2, and TC + KO_2 groups at baseline and AITC-induction timepoints. Results are means ± SEM of four culture dishes per group and each culture dish was a pool of DRG from 5 mice (n=4 culture dishes). Statistical analyses were performed by two-way ANOVA followed by Tukey's multiple comparisons test (E). *p-value ≤ 0.05 vs. vehicle + saline group; #p-value ≤ 0.05 vs. vehicle + KO_2 group.

Discussion

In this study, we evaluated the activity of TC to protect against ROS-triggered inflammation and pain. The TC anti-inflammatory and analgesic mechanisms targeted redox balance and inflammatory pathways that are linked by Nrf2 and NF-κB as well as the sensitization of TRPV1$^+$ and TRPA1$^+$ peripheral nociceptive neurons. These mechanistic studies explain the anti-inflammatory and analgesic activities of TC in a model in which ROS trigger an inflammatory painful response as we will discuss in detail.

ROS modulate multiple physiological and pathological functions such as cell proliferation, differentiation, death, and migration.[4] More specifically, the superoxide anion is produced by leaking out of mitochondrial respiratory chain as well as by NADPH oxidase[42] or as a by-product of enzymes such as COX-2. Superoxide anion triggers an inflammatory process.[43] Interestingly, KO_2 induces a transient release of superoxide anion that lasts for at least 5 min.[18] However, hours after KO_2 injection, superoxide anion is still produced locally, a response that depends, at least in part, on the activity of NADPH oxidase.[24,44] Evidence supports that the transcription factor NF-κB is responsible for up-regulating NADPH oxidase gp91^{phox} expression.[9,45] Interestingly, NF-κB is a transcription factor that is activated by ROS such as superoxide anion and hydrogen peroxide indicating it as part of an auto-feedback fueling loop.[4,7,40] Therefore, it is likely that KO_2 releases superoxide anion to induce oxidative stress, which is boosted by triggering NF-κB activation that up-regulates gp91phox mRNA expression, allowing endogenous NADPH oxidase-dependent additional production of superoxide anion. This excessive ROS production will lead to lipid peroxidation and tissue damage.[9,10] TC inhibited COX-2 and gp91phox mRNA expression and phosphorylation of NF-κB with consequent reduction of superoxide anion production and lipid peroxidation. Adding further complexity to the TC activity, the overall antioxidant effect of TC appears to also be dependent on the induction of endogenous protective mechanisms. TC increased the mRNA expression of Nrf2, a transcription factor sensitive to redox balance that up-regulates the production of endogenous antioxidants. In fact, TC increased the tissue ability to reduce iron and scavenge the radical ABTS, demonstrating an increase of endogenous protective antioxidant defenses.

Notably, TC is not an antioxidant per se.[26] Its chemical structure lacks hydroxyl groups, which would allow donating hydrogen to accept electrons, thus allowing an antioxidant activity.[46] Therefore, the observed antioxidant effects were not dependent on the expected antioxidant activity as for most regular antioxidant flavonoids. This is also important because we can speculate that flavonoids induce antioxidant responses in vivo that are not solely dependent on structural antioxidant properties, as a flavonoid that is not an antioxidant per se can trigger in vivo antioxidant signaling programs that will inhibit oxidative stress, while in vitro in cell-free systems, no antioxidant activity is observed for TC.[26]

TC pre-treatment reduced edema and neutrophil/macrophage recruitment; the latter was assessed indirectly by myeloperoxidase activity, an enzyme found mainly, but not exclusively, in lysosomal azurophilic granules of neutrophils recruited during the initial hours of acute tissue inflammation.[47] Circulating monocytes/macrophages that are recruited to the inflammatory foci also express the myeloperoxidase.[48] Previous studies have shown TC ability to inhibit the adhesion molecules ICAM-1 (intercellular adhesion molecule 1), and VCAM-1 (vascular cell adhesion molecule 1) in a cardiac ischemia and reperfusion experimental study,[49] indicating that it could be a mechanism contributing to the inhibition of leukocyte recruitment. In addition, superoxide anion is important for the guidance of neutrophils to the inflammatory foci; therefore, inhibiting their production also interferes with the recruitment of these phagocytes.[50,51]

It is noteworthy to discuss that Nrf2 and NF-κB pathways present some interactions. These transcription factors are both sensitive to the cellular redox signaling, although they have different functions. NF-κB enhances the production of proinflammatory molecules and of molecules involved in the induction of oxidative stress. Nrf2 stimulates antioxidant pathways to limit oxidative stress and reduce proinflammatory molecules.[52] Interestingly, NF-κB and Nrf2 compete for binding to the transcriptional co-activator CREB-binding protein (CBP/p300). Their ability to induce gene transcription depends on binding to CBP/p300, and an increase in one will reduce the activity of the other, although the affinity of NF-κB for CBP/p300 is higher than that of Nrf2.[53] In addition, Nrf2 limits redox activation of NF-κB.[54] Therefore, the ability of TC to increase Nrf2 mRNA expression and reduce p-NF-κB is consistent with the overall reduction in oxidative stress and inflammatory molecules.

Superoxide anion induces the activation of NF-κB, which regulates the downstream production of cytokines (eg., IL-1β, TNFα, IL-6, and IL-33)[9] and COX-2 expression,[7] an enzyme involved in prostanoid production. Cytokines and prostanoids are involved in the development of inflammation and pain.[55] For instance, the inhibition of cytokines (eg., IL-1β, TNFα, IL-6, and IL-33) reduces neutrophil recruitment, edema formation, and hyperalgesia.[9] This cytokine-mediated mechanism is important in various diseases such as arthritis and colitis.[31,56] Cytokines are also involved in nociceptive neuron sensitization and activation, mediating both non-evoked nociceptive behaviors such as abdominal contortions[33] and mechanical hyperalgesia.[13] Cytokines and prostanoids sensitize nociceptive sensory neurons by increasing the firing and opening of ion channels, facilitating the depolarization and transmission of nociceptive

input.[57] In acute inflammation, the cytokine effect is mostly indirect and dependent on the production of COX-2-derived prostaglandin E_2, which sensitizes nociceptive neurons and causes hyperalgesia.[13] TC also inhibited the production of IL-10, which is widely accepted as an anti-inflammatory and analgesic cytokine in most diseases. IL-10 is co-released with pro-inflammatory cytokines with a role of balancing the inflammatory response. In agreement with this concept, deleting IL-10 exacerbates the inflammatory response because it has this endogenous role of counterbalancing/limiting the inflammatory response. When a treatment reduces the production of pro-inflammatory cytokines, sometimes it also reduces the release of IL-10 because it is not necessary to release IL-10 to counterbalance the inflammation.[58–60] This role of IL-10 resembles in part what is observed with endogenous corticoids that also have a role in limiting inflammation. Their absence, for instance, by an adrenalectomy, results in exacerbated inflammation.[61]

The TRP channels present in the membranes of nociceptive neurons include the TRPV1 and TRPA1. The expression of TRPV1 is characteristic of peripheral afferent unmyelinated type C nociceptive neurons.[62] Enhancement of activity and expression of TRPV1 are mechanisms of nociceptive neuron sensitization. TRPV1 is involved in sensing thermal stimulation and pH extremes, acidic, and basic microenvironments.[63] It has been demonstrated for some flavonoids that they inhibit TRPV1 signaling, including vitexin,[34] hesperidin methyl chalcone,[64] and eriodictyol.[65] TRPA1 is also expressed by C fibers and is involved in sensing mechanical and cold stimuli.[66] TRPA1 channels also sense ROS through cysteine oxidation and disulfide bond formation between TRPA1 cysteine residues.[67] TRPA1 ion channels are expressed by Schwann cells, oligodendrocytes, astrocytes, primary afferent neurons, vascular endothelial cells, and other tissues that can relay nociceptive signals, indicating that the inhibition of TRPA1 may represent a mechanism of action with broader consequences.[68]

Our research group has recently shown that TC is able to inhibit nociceptive signaling triggered by TRPV1 and TRPA1 in vivo using overt pain-like behavior models induced by acetic acid, phenyl-*p*-benzoquinone (PBQ), formalin, complete Freund's adjuvant (CFA), and the TRP channel agonists capsaicin (TRPV1) and allyl isothiocyanate (AITC, TRPA1). In vitro, TC also inhibited the neuronal activation caused by capsaicin and AITC in primary culture of naïve DRG-derived neurons, which demonstrates TC inhibits the neuronal activation of TRPV1 and TRPA1.[32] Here, we demonstrate that TC analgesic effects are also observed in an experimental model of pain triggered by oxidative stress. Superoxide anion-triggered nociceptive behavior was inhibited by TC as well as targeting TRPV1 and TRPA1 ion channels with selective antagonists (AMG-9810 and HC-030031, respectively).

In this study, KO_2 injection in the paw triggered an increase in calcium influx in the DRG neurons. The response of KO_2 group-derived DRG neurons to capsaicin and AITC was higher than that of saline group-derived DRG neurons. This higher response of KO_2 group-derived DRG neurons occurs because of neuronal sensitization with the enhancement of TRPV1 and TRPA1 responsiveness.[63,69–71] TC treatment in vivo reduced basal calcium levels in DRG neurons of mice with KO_2 triggered inflammation demonstrating that the analgesic effect of this flavonoid assessed by behavioral assays is also supported by a neuronal functional response assay. TRPA1 inhibition by TC might also be mechanistically involved in inflammation, because both genetic ablation and pharmacological inhibition of TRPA1 reduce skin edema, and TRPA1 is expressed by a great variety of non-neuronal cells, as mentioned above.[72]

It is important to discuss the limitations of the present study. The present experimental design was not intended to perform dose–response curves. We selected the TC dose accordingly to previous data from our laboratory[31] but it is a limitation. Our focus was on the mechanisms of action of TC. There is no experimental pharmacokinetic data in the present study. However, applying the SwissADME – a platform to predict absorption, distribution, metabolism, and excretion,[73] the TC bioavailability score is 0.55 meaning favorable oral absorption, typically indicating that roughly >10% will likely be bioavailable in the systemic circulation. SwissADME also indicates relatively high gastrointestinal absorption and to be blood–brain barrier permeable.[74] This is in accordance with the observed biological activity of TC upon P.O. administration. There is also the possibility of developing pharmaceutical delivery systems to improve TC administration, absorption, delivery, and activity,[67] which would increase the probability of TC to reach clinical use. We do not present data on possible metabolization of TC that could lead to additional bioactive or inactive derivatives in vivo. However, this limitation does not alter the interpretation of the present findings since the entire experimental approach is in vivo. If there were in vitro treatments with TC, this could raise the question of whether TC would indeed be the main bioactive molecule, even though there is no evidence to our knowledge that metabolization is required for its

activity. Body surface area (BSA) normalization is considered an accurate form to estimate the human equivalent dose (HED) when taking preclinical studies data to calculate probable human doses.[75] Using BSA approach,[75] we would have a 2.43 mg/kg dose for humans. For a human with 70kg, this would be 170.1 mg of TC. Thus, this projection suggests that it is possible to achieve a TC dose to obtain pharmacological activity in humans. Another point is that the current study did not present data further investigating whether targeting Nrf2 would reduce the activity of TC. Despite these limitations, this study adds to the understanding of TC activity and mechanism of action.

Conclusions

Flavonoids are known for their ability to reduce oxidative stress, which is explained by the inherent antioxidant activity due to their chemical structure that allows hydrogen donation and electron acceptance. This characteristic brings the challenge of investigating possible signaling modulation induced by flavonoids that could occur beyond their intrinsic antioxidant characteristics. Herein, we applied an atypical flavonoid, TC, that lacks an intrinsic antioxidant structural activity, to investigate flavonoid mechanisms that are independent on their antioxidant structural activity. Pain and inflammation were experimentally induced by a superoxide anion donor, KO_2, to introduce the challenge of inhibiting inflammation and pain that are dependent on oxidative stress. The present data demonstrate that TC reduces inflammation and pain even in the absence of chemical groups to confer structural antioxidant properties. TC reduced NF-κB activation and increased Nrf2 mRNA expression. As discussed above NF-κB and Nrf2 are two transcription factors that are redox sensitive, regulate the expression of molecules involved in inflammation and oxidative stress and share co-transcription activators. TC also presents neuronal effects adding to explain its analgesic activity by inhibition of KO_2 triggered activation of $TRPV1^+$ and $TRPA1^+$ neurons. Therefore, despite its atypical chemical structural characteristics that would lead to the assumption of low bioactivity potential compared to other flavonoids, on the contrary, TC is a promising flavonoid with multitarget mechanisms of action to reduce inflammation and pain in diseases with a role of ROS such as superoxide anion.

Data Sharing Statement

Data will be made available upon reasonable request to corresponding authors.

Ethical Approval

The Animal Ethics Committee of Londrina State University (processes 24684.2016.72, December 8, 2016, and 050.2024, December 12, 2024) approved all the study procedures. All efforts were made to minimize the number of animals used and their suffering. Animal studies were reported in compliance with the ARRIVE guidelines and IASP recommendations.

Author Contributions

Maiara Piva; Data curation, Formal analysis, Investigation, Visualization, Writing – original draft. Writing – review and editing. Marília F. Manchope; Data curation, Formal analysis, Investigation, Writing – original draft. Fernanda Barbosa-Costa; Data curation, Formal analysis, Investigation, Writing – review and editing. Beatriz H. S. Bianchini; Data curation, Formal analysis, Investigation, Writing – review and editing. Ketlem C. Andrade; Data curation, Formal analysis, Investigation, Writing – review and editing. Letícia Coelho Silva; Data curation, Formal analysis, Investigation, Writing – review and editing. Cássia Calixto-Campos; Data curation, Formal analysis, Investigation, Writing – review and editing. Fernanda S. Rasquel-Oliveira; Data curation, Formal analysis, Investigation, Writing – review and editing. Victor Fattori; Conceptualization, Data curation, Formal analysis, Investigation, Funding acquisition, Writing – original draft. Ana Carla Zarpelon-Schutz; Data curation, Formal analysis, Investigation, Writing – original draft. Doumit Camilios-Neto; Conceptualization, Data curation, Formal analysis, Funding acquisition, Writing – original draft. Sergio M. Borghi; Conceptualization, Data curation, Formal analysis, Funding acquisition, Writing – original draft, Writing – review and editing. Rubia Casagrande; Conceptualization, Data curation, Formal analysis, Funding acquisition, Supervision, Writing – original draft, Writing – review and editing. Waldiceu A. Verri; Conceptualization, Data curation, Formal analysis, Funding acquisition, Supervision,

Writing – original draft, Writing – review and editing. All authors gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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