Neutrophil-derived COX-2 has a key role during inflammatory hyperalgesia

Nathalia Santos Carvalho
University of Campinas
Julia Borges Paes Lemes
University of Campinas
Marco Pagliusi Jr
University of Campinas
Ana Carolina Machado
University of Campinas
Kauê Franco Malange
University of Campinas
Lais Passariello Pral
University of Campinas
José Luís Fachi
University of Campinas
Catarine Nishijima Massucato
University of Campinas
Gilson Gonçalves dos Santos
University of California, San Diego
Claudia Herrera Tambeli
University of Campinas
Cesar Renato Sartori
University of Campinas
Marco Aurélio Ramirez Vinolo
University of Campinas
Carlos Amilcar Parada (caparada@unicamp.br)
University of Campinas

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Abstract

Inflammation is a vital process for the injured tissue restoration and one of its hallmarks is inflammatory hyperalgesia. The cyclooxygenase (COX) pathway is strongly related to the inflammatory and painful process. Usually, the COX-1 isoform is described as homeostatic, while COX-2 is characterized as inducible in inflammatory conditions. Although it is well known that neutrophil cells are the first to arrive at the inflamed site and the major source of COX-2 is still unknown the specific role of neutrophil-derived Cox-2 in the pain process is. Thus, in the present study, we demonstrate for the first time that neutrophil-derived COX-2 plays a key role in peripheral inflammatory hyperalgesia. Conditional knockout mice for Cox-2 in neutrophils (Cox-$2^{fl/fl: Mrp8cre+/-}$) exhibited higher pain sensitivity after carrageenan (CG) injection and long-lasting IL-1β-induced hyperalgesia compared with the control group (COX-$2^{fl/fl}$). Also, CG-induced inflammation in Cox-$2^{fl/fl: Mrp8cre+/-}$ mice showed Cox-1 overexpression, and increased neutrophil migration and pro-inflammatory cytokines (*e.g.*, IL-1β and CXCL1). These findings revealed that neutrophil Cox-2 has an important role in the regulation of inflammatory hyperalgesia.

Introduction

Tissue injury promotes an inflammatory process associated with a series of cellular and molecular events linked with hyperalgesic states [1]. Inflammation is an important biological process that aims to eliminate the damaging stimulus and promote tissue repair rapidly and control [2]. This process may eventually undergo deregulation and present a prolonged and exacerbated action, resulting in persistent inflammation, and delayed tissue repair, contributing to chronic disease conditions[3].

In the acute phase of inflammation, neutrophils are described as one of the primary cell-type involved and act as the first line of defense in eliminating pathogens [4, 5]. In this context, neutrophils regulate inflammatory processes through their toxic arsenal of damaging pathogen molecules, and the synthesis of cytokines, such as interleukin (IL)-1β, tumor necrosis factor (TNF)-α, and CXCL-1 and cyclooxygenases (COX's), by which they can also be influenced [6, 7].

The COX-1 and COX-2 isoenzymes play an essential role in several biological processes. These enzymes convert the arachidonic acid into prostaglandins (PGs) [8, 9]. The isoform COX-1 is predominantly constitutive in the cells and essential to homeostasis and housekeeping activities [10]. However, its role in inflammatory processes is still poorly understood. On the other hand, inducible isoform COX-2 plays a crucial role in inflammation and inflammatory pain. Different immune system cells, such as macrophages [11] and neutrophils, express the COX-2 isoenzyme [12, 13]. Neutrophils' Cox-2 activation results in PGE_2 synthesis that, in addition to its function in inflammatory pain [14], may also be important to the inflammation resolution [15, 16].

Coxibs are known as 'selective Non-Steroidal Anti-Inflammatory Drugs (NSAIDs)' once they only act inhibiting the COX-2 isoenzyme[17]. The COX-2 selective inhibition was designed to minimize gastrointestinal complications, which is an important side effect of Cox-1 inhibition by non-selective

NSAIDs[18]. Nonetheless, controlled trials revealed that long-term use of coxibs significantly increases the risk of myocardial infarction and stroke, as well as being associated with skin reactions[19]. In this context, the importance of understanding the role of COX-2 not just as a pro-inflammatory agent but also in its role in homeostatic processes is evident.

Although it is well known that neutrophil cells are the first to arrive at the inflamed site and the major source of COX-2 is still unknown the specific role of neutrophil-derived Cox-2 in the pain process is. Thus, in the present study, using a neutrophil-COX-2 knocked out mice (Cox-$2^{fl/fl: Mrp8cre+/-}$), we aim to analyze the role of neutrophil-COX-2 in i) inflammatory pain, ii) cell migration, and iii) cytokine releasing.

Methodology

Drugs

This study used the following reagents: Carrageenan (CG) 1% 25uL/paw Sigma Aldrich (St. Louis, MO, USA); IL-1β 1 pg/paw Farmaformula® (Fagron, Brazil); Fucoidan (FC) 50 mg/kg Sigma Aldrich (St. Louis, MO, USA); Valeryl Salicylate (VS) 50 mg/kg Sigma Aldrich (St. Louis, MO, USA). All drugs were dissolved in 0,9% saline (Veh). The other chemicals used in the present work were of analytical grade and obtained from standard commercial suppliers.

Animals

In the present study mice with loxP sites flanking exon 4 and 5 (Cox-$2^{flox/flox}$ mice) [20] were crossed to mice expressing Cre recombinase under the control of the human Mrp8 promoter (Mrp8creTg), in which Cre activity is restricted to neutrophils. Conditional knockout mice for Cox-2 in neutrophils (Cox-$2^{fl/fl: Mrp8cre+/-}$) allow us to address questions regarding the neutrophil-specific role of COX-2 in inflammatory hyperalgesia. Cox-$2^{fl/fl}$ mice were used in the present study as the control group, as previously described (An et al., 2014; Escuin-Ordinas et al., 2014).

Eight-week-old male and female (25-30 g) mice were used, with free access to water and a standard diet. They were housed in cages in a controlled temperature environment and 12h/12h light-dark cycle. All behavioral experiments were conducted in a randomized and double-blinded manner.

CG-induced inflammatory hyperalgesia and Pharmacological modulation of the inflammatory hyperalgesia.

Mice received intraplantar administration (25 µl/paw) of 1% CG or 0,9% Veh (Veh group) one hour before the behavioral test or IL-1β (1pg /25 µl/paw) 20 minutes before the behavioral test. We administered all drugs in the hind paw using a hypodermic 26-gauge needle coupled with a 50 µL Hamilton syringe to administer 25 µl.

We evaluated the influence of neutrophil migration and the role of COX-1 *in vivo* on CG-induced inflammatory hyperalgesia by the intraperitoneal (i,p) administration of FC (leukocyte migration inhibitor)

or VS (cox-1 specific inhibitor), both 50 mg/kg in 200 µL. We performed all injections using a 30G needle connected to an insulin syringe (1mL), and the formulations were administered 30 minutes before the CG 1% injection in the paw; the control group received 0,9% saline. We used a different group of mice for ELISA analysis. The plantar tissue of this group was dissected and collected at the third hour after CG (peak of CG action) or saline administration.

Electronic von Frey

The mechanical hyperalgesia threshold of the mice's hind paws was assessed by the adapted electronic von Frey test (EFF 301 Digital Analgesiometer, Insight, Brazil) as previously described [22]. A gradual force was applied with a 0.5 mm diameter polypropylene disposable tip to the central part of the mice's hind paw until it performs a withdrawal reflex, followed by a response characterized as limb tremor (flinch). The transduction of the force applied to the mice's paw was measured in grams (g) by a digital force counter, and we considered a valuable response after three similar measurements with a 10% margin error. The animals were initially adapted 15 to 30 minutes before the test, for environmental adaptation, in a quiet room and arranged individually in acrylic boxes (12 x 10 x 17 cm) with a 1mm thick non-malleable wire mesh floor and spaces of 5mm. The hyperalgesia intensity corresponds to the post-treatment measurement's subtraction from the baseline values (Δ mechanical threshold).

Real-time (RT)-PCR

We extracted the total DNA from the plantar tissue of mice using Trizol® reagent (Invitrogen, USA). According to the manufacturer, the cDNA synthesis was performed with the High Capacity cDNA synthesis kit (Applied Biosystems, Foster City, CA, USA). We determined the COX-1 e COX-2 gene expression on a StepOnePlus® Applied PCR system using the SYBR® Green PCR Master Mix (Applied Biosystems, USA). We used GAPDH gene expression as housekeeping. Exxtend® supplied all primers. See **Table I** for the primer sequences.

Cytokine measurements

We measured the levels of TNF-α, IL-1β, IL-10, and CXCL1 in plantar tissue by an enzyme-linked immunosorbent assay (ELISA), Kit DuoSet (R&D Systems), according to the manufacturer instructions. We determined the levels of cytokines by comparing the samples' optical density to the standard curve's densities and expressed them as milligrams of each cytokine per milliliter (mg/mL). The results were normalized by protein quantity in the sample by Bradford assay.

MPO activity

We assessed the neutrophil migration by evaluating Myeloperoxidase (MPO) activity according to the method described [23] with modifications, using plantar tissue samples. The resulting supernatant was assayed for MPO activity spectrophotometrically at 450 nm, and the results were expressed as a unit of MPO per mg of tissue (UMPO/mL tissue). The measurement unit of MPO activity was defined by the

amount of MPO capable of catalyzing the peroxidation reaction of 10 millimoles of H2O2 in 1 minute at 25 degrees Celsius.

Flow cytometry

We harvested peritoneal cells from Cox-2$^{fl/fl}$ and Cox-2$^{fl/fl: Mrp8cre+/-}$ mice 4 hours after i.p administration of CG 1% or PBS. Cells were then washed, resuspended in 100 µL of FACS buffer (BD Biosciences), and incubated for 20 minutes with antibodies to analyze the immune cells' population and frequency. Cell acquisition was performed on FACSymphony™ A5 High-Parameter Cell Analyzer(BD Biosciences) using FACSDiva software (BD Biosciences), and all data were analyzed using FlowJo software (TreeStar Inc.; Ashland, OR, USA).The following antibodies were used: PE-Cy7CD45 (clone 104),FITC Ly-6G (clone 1A8), APCCD11b (M1/70), PECD11c (N418), and APC-Cy7 F4/80 (clone BM8)(Biolegend, USA). We discriminated dead cells from live cells using LIVE/DEAD fixable dead stain (BD Biosciences) and all staining steps carried out in media containing anti-CD16/32 (2.4G2 antibody).

Statistical analysis

The statistical analysis was performed by Prism v.6 (GraphPad Software) using the One and Two-way Analysis of Variance test (ANOVA), followed respectively by Tukey or Bonferroni's post-test, according to the number of parameters compared for each experiment. A student's *t*-test was performed to determine the level of statistical significance between the two groups. All data were expressed as mean ± SD, and differences were considered significant when $p \leq 0.05$.

Results

Mice lacking COX-2 in neutrophil has an exacerbated pain profile.

Figure 1A shows the intensity of mechanical hyperalgesia (variation of the threshold) 1h, 2h, 3h, 4h, 5h, 6h, and 24h after CG injection Cox-2$^{fl/fl}$ and Cox-2$^{fl/fl: Mrp8cre+/-}$. Two-way ANOVA showed significant differences between groups ($F_{7, 91}=12.93$; $p \leq 0.0001$) and time ($F_{7, 91}=166.1$; $p \leq 0.0001$). The *post hoc* Bonferroni's multiple comparison test revealed statistical differences between groups 1h ($p \leq 0.05$), 2h, 3h, 4h, 5h, and 6h ($p \leq 0.001$) except for 24h following the CG injection, showing that neutrophil Cox-2$^{fl/fl: Mrp8cre+/-}$ mice display more intense CG-induced hyperalgesia. This effect can also be observed by analyzing the area under the curve ($p \leq 0.001$), showed in **figure 1B**. Note that 3h after CG injection we observe the peak of hyperalgesia in both groups and, because of this, we use this time point for all following experiments. **Figure 1C** shows the intensity of hyperalgesia 3h after CG injection for male and female, showing that both Cox-2$^{fl/fl:Mrp8cre+/-}$ gender display more intense CG-induced hyperalgesia (unpaired T-test; male: $t=8.800$ $df=13$, $p \leq 0.0001$; female: $t=3.974$ $df=10$, $p = 0.0026$). It is important to mention that there is no statistical difference between males and females from the same group.

<Figure 1 near here>

The lack of COX-2 in neutrophils triggers an over-release of pro-inflammatory cytokines during inflammation

After observing higher inflammatory pain intensity in Cox-$2^{fl/fl:Mrp8cre+/-}$ group, we decided to evaluate the profile of pro-inflammatory cytokines released in the inflamed tissue of these mice. **Figure 2A-D** shows the level of cytokines released after Veh or CG injection in the hind paw for Cox-$2^{fl/fl}$ and Cox-$2^{fl/fl:Mrp8cre+/-}$ groups. We can observe that Cox-$2^{fl/fl:Mrp8cre+/-}$ mice over-release CXCL-1 and IL-1β when compared to Cox-$2^{fl/fl}$. **Figure 2A** shows the CXCL-1 released level for both groups. One-way ANOVA showed significant differences between groups ($F_{3,20}$ =27.83; $p \leq 0.0001$). The *post hoc* Tukey`s multiple comparison test revealed statistical differences between Cox-$2^{fl/fl}$ Veh and Cox-$2^{fl/fl}$ CG ($p \leq 0.01$); also, there is statistical differences between Cox-$2^{fl/fl:Mrp8cre+/-}$ Veh and Cox-$2^{fl/fl:Mrp8cre+/-}$ CG ($p \leq 0.001$). Additionally, it is important to highlight the significant differences in CXCL1 release between the Cox-$2^{fl/fl}$ and Cox-$2^{fl/fl:Mrp8cre+/-}$ CG groups ($p \leq 0.001$), (Cox-$2^{fl/fl}$: Veh = 14.18 ± 2.268mg/mL; CG = 79.91 ± 11.98mg/mL; Cox-$2^{fl/fl:Mrp8cre+/-}$: Veh = 46.48 ± 8.081mg/mL; CG = 162.3 ± 19.19mg/ mL). **Figure 2B** shows the IL-1β released level for both groups. One-way ANOVA showed significant differences between groups ($F_{3,15}$ =71.30; $p \leq 0.0001$). The *post hoc* Tukey`s multiple comparison test revealed statistical differences between Cox-$2^{fl/fl}$ Veh and Cox-$2^{fl/fl}$ CG ($p \leq 0.05$); also, there is statistical differences between Cox-$2^{fl/fl:Mrp8cre+/-}$ veh and Cox-$2^{fl/fl:Mrp8cre+/-}$ CG ($p \leq 0.001$), and once again, we highlight the differences in IL-1β release between the Cox-$2^{fl/fl}$ and Cox-$2^{fl/fl:Mrp8cre+/-}$ CG groups ($p \leq 0.001$) (Cox-$2^{fl/fl}$: Veh = 517.2 ± 105.3mg/mL; CG = 1773.00 ± 332.6mg/mL; Cox-$2^{fl/fl:Mrp8cre+/-}$: Veh = 355.0 ± 92.33mg/mL; CG = 5532.0 ± 469.4mg/mL). **Figure 2C** shows the TNFα level for both groups. One-way ANOVA showed no significant differences between groups ($F_{3,20}$ =1.744; $p \leq 0.1904$) (Cox-$2^{fl/fl}$: Veh 25.61 ± 4.405mg/mL; CG = 16.82 ± 3.328mg/ mL; Cox-$2^{fl/fl:Mrp8cre+/-}$: Veh = 32.34 ± 6.803mg/mL; CG = 23.72 ± 4.088mg/mL). **Figure 2D** shows the IL-10 released level for both groups. One-way ANOVA showed no significant differences between groups ($F_{3,16}$ = 3.053; $p \leq 0.05$). (Cox-$2^{fl/fl}$: Veh=0.121 ± 0.011 mg/mL; CG = 0.083 ± 0.016 mg/mL; Cox-$2^{fl/fl:Mrp8cre+/-}$ Veh = 0.097 ± 0.015 mg/mL; CG= 0.0595 ± 0.014 mg/mL).

<Figure 2 near here>

Mice lacking COX-2 in neutrophils showed long-lasting hyperalgesia following IL-1β injection.

Considering that Cox-$2^{fl/fl: Mrp8cre+/-}$ mice over-release IL-1β – a key cytokine for inflammatory hyperalgesia – during an inflammatory process, we tested if injecting IL-1β in the hind paw would trigger an exacerbated hyperalgesia in these mice. Our data showed that Cox-$2^{fl/fl: Mrp8cre+/-}$ mice display long-lasting hyperalgesia when compared to Cox-$2^{fl/fl}$ mice. **Figure 3A** shows the intensity of hyperalgesia at 20 min, 40 min, 60 min, 90 min, 120 min, 150 min, and 180 min after IL-1β injection for Cox-$2^{fl/fl}$ and Cox-$2^{fl/fl: Mrp8cre+/-}$ mice. Two-way ANOVA showed significant differences between groups (F6,54=21.23; $p \leq 0.0001$) and time (F6,54=177.5; $p \leq 0.0001$). The *post hoc* Bonferroni's multiple comparison test revealed statistical differences between groups at two time points 150 min and 180 min after IL-1β injection ($p \leq$

0.001). Figure 3B shows the area under the curve (AUC), showing an expressive increase in the hyperalgesia for Cox-2$^{fl/fl: Mrp8cre+/-}$ mice ($p \leq 0.001$).

<Figure 3 near here>

COX-2 contributes to neutrophil migration during inflammatory pain.

Considering that leukocyte migration is necessary for the inflammatory hyperalgesia, we investigated the levels of neutrophil migration in CG-induced inflammation by MPO quantification in the hind paw and leukocyte migration by cytometry in the peritoneal washed. **Figure 4A** shows the quantity of MPO in the hind-paw tissue after Veh or CG injection. Our data showed a MPO increase in both groups after CG injection when compared to Veh ($p \leq 0.001$; t=4.586 df=8 and t=7.341 df=7). We also demonstrated that Cox-2$^{fl/fl:Mrp8cre+/-}$ mice showed higher CG-induced MPO increase ($p \leq 0.001$; t=4.604 df=8) when compared to Cox-2$^{fl/fl}$ mice (Cox-2$^{fl/fl}$: Veh = 2.294 ± 0.279 U; CG = 4.432 ± 0.373U; Cox-2$^{fl/fl:Mrp8cre+/-}$: Veh = 2.050 ± 0.194U; CG = 8.158 ± 0.718U). We further investigate the cell migratory profile for both groups during inflammation. **Figure 4C-E** shows the peritoneal percentage of Ly6G^{+}CD11b^{+} neutrophils, CD11b^{+}F4/80^{+} macrophages, and CD11b^{+}CD11c^{+} dendritic cells on CD45^{+} compartment after CG or Veh injection, respectively. **Figure 4B** shows the representative flow cytometry dot plots of immune cells in peritoneal washing after Veh or CG *i.p.* injections. **Figure 4C** shows the percentage of neutrophils in peritoneal washing after Veh or CG *i.p.* injections for both groups. Our data showed that Cox-2$^{fl/fl}$ ($p \leq 0.01$; t=3.630 df=15) and Cox-2$^{fl/fl:Mrp8cre+/-}$ ($p \leq 0.01$; t=3.229 df=15) mice showed higher neutrophil infiltrate after CG injection when compared to Veh (Cox-2$^{fl/fl}$: Veh = 13.16 ± 3.571; CG = 18.74 ± 1.689; Cox-2$^{fl/fl:Mrp8cre+/-}$: Veh = 23.80 ± 1.554; CG = 34.28 ± 4.305). We also demonstrated that Cox-2$^{fl/fl:Mrp8cre+/-}$ mice exhibit higher neutrophil infiltrate after Veh ($p \leq 0.01$; t=3.722 df=14) and CG ($p \leq 0.001$; t=4.545 df=16) stimulus when compared to Cox-2$^{fl/fl}$ mice. **Figure 5D** shows the percentage of macrophages in peritoneal washing after Veh or CG *i.p.* injections for both groups. Our data showed no difference between groups (Cox-2$^{fl/fl}$: Veh = 10.32 ± 1.318; CG = 12.76 ± 2.276; Cox-2$^{fl/fl:Mrp8cre+/-}$: Veh = 8.852 ± 1.778; CG = 10.33 ± 2.407). **Figure 5E** shows the percentage of dendritic cells in peritoneal washing after Veh or CG *i.p.* injections for both groups. Our data showed no difference between groups (Cox-2$^{fl/fl}$: Veh = 3.356 ± 0.991; CG = 2.934 ± 0.271; Cox-2$^{fl/fl:Mrp8cre+/-}$: Veh = 2.512 ± 0.236; CG = 2.414 ± 0.216).

<Figure 4 near here>

CG-induced hyperalgesia in mice lacking COX-2 in neutrophils is dependent on neutrophil migration.

Considering the higher leukocyte migration in Cox-2$^{fl/fl:Mrp8cre+/-}$ mice, we tested if FC – a leukocyte migration inhibitor – would blocks the inflammatory hyperalgesia even in the Cox-2 absence in this cell-type. **Figure 5** shows the intensity of hyperalgesia 3h after CG + Veh or CG + FC injection. Our data showed reduction in hyperalgesia intensity in both, Cox-2$^{fl/fl}$ and Cox-2$^{fl/fl:Mrp8cre+/-}$ FC groups when compared to CG groups, which demonstrates that leukocyte migration is necessary for inflammatory

hyperalgesia regardless Cox-2 expression (Cox-$2^{fl/fl}$: CG = 3.520 ± 0.276; FC = 0.620 ± 279; *p*< 0,0001; t=7.384 df=8; Cox-$2^{fl/fl:Mrp8cre+/-}$: CG = 6.226 ± 0.586; FC = 1.478 ± 0.303; *p*< 0.0001; t=7.192 df=8).

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Mice lacking COX-2 in neutrophil shows *cox-1 overexpression* in the hind pawn after CG injection.

Once we observed an increase in inflammatory hyperalgesia intensity in mice lacking Cox-2 in neutrophils, we investigated if Cox isoenzymes would be differently expressed in the inflamed tissue of these mice. **Figure 6A** shows *cox-1* and *cox-2 mRNA* expression in the hind pawn tissue for Cox-$2^{fl/fl}$ and Cox-$2^{fl/fl:Mrp8cre+/-}$ mice after Veh or CG injection. Our data shows no difference (*p* = 0.258) in *cox-1* expression but statistical difference (*p*< 0.0001) in *cox-2* expression between Veh and CG for Cox-$2^{fl/fl}$ mice (*cox-1:* Veh = 1.024 ± 0.1159; CG = 1.334 ± 0.2272; t=1.215 df=8; *cox-2:* Veh = 1.042 ± 0.1578; CG = 6.600 ± 0.6714; t=8.059 df=8). For Cox-$2^{fl/fl:Mrp8cre+/-}$ mice our data shows overexpression (*p* = 0.0067) of *cox-1* after CG injection when compared to Veh, and almost undetected *cox-2* expression (*cox-1:* Veh= 1.011 ± 0.068; CG = 1.467 ± 0.117; t=3.505 df=9; *cox-2:* Veh = 0.0179 ± 0.0137; CG = 0.0494 ± 0.029; t=0.980 df=8; *p* = 0.355).

Following this result, we hypothesized that another COX isoenzyme could be signaling replacing COX-2. We confirmed this hypothesis using VS, a specific COX-1 inhibitor, which reduced the inflammatory hyperalgesia in Cox-$2^{fl/fl:Mrp8cre+/-}$ mice, but not in Cox-$2^{fl/fl}$. **Figure 5B** shows the intensity of hyperalgesia 3h after CG + Veh or CG + VS injection. Our data showed reduction in hyperalgesia intensity in Cox-$2^{fl/fl:Mrp8cre+/-}$ VS group but not in Cox-$2^{fl/fl}$ when compared to CG group (Cox-$2^{fl/fl}$: CG = 3.682 ± 0.175; VS = 4.620 ± 0.324; *p* < 0,05; t=2.545; df=8; Cox-$2^{fl/fl:Mrp8cre+/-}$: CG = 4,474 ± 0,409; VS = 2,594 ± 0,284; *p* < 0,01; t=3,771; df=8). Thus, these findings revealed the importance of neutrophils in inflammatory hyperalgesia even in the absence of neutrophil-derived Cox-2, in addition, at least in part, Cox-1 may be mediating hyperalgesia in Cox-$2^{fl/fl:Mrp8cre+/-}$ mice.

<Figure 6 near here>

Mice lacking COX-2 in neutrophil shows *cox-1 over*expression and undetected expression of *cox-2* after LPS stimulus *in vitro.*

Aiming to confirm our cell-specific knock out model, we isolated neutrophils from bone marrow and stimulated these cells using LPS *in vitro*, aiming to mimic an inflammatory environment. **Figure 7** shows *cox-1* and *cox-2* mRNA expression in neutrophil culture from Cox-$2^{fl/fl}$ and Cox-$2^{fl/fl:Mrp8cre+/-}$ mice after LPS stimulus. Our data showed that Cox-$2^{fl/fl:Mrp8cre+/-}$ exhibit *cox-1* overexpression when compared to Cox-$2^{fl/fl}$ ($p \leq 0.001$; t=13.45 df=8), and, as expected, undetected expression of *cox-2* (cox-$2^{+/+}$: *Cox 1* = 1.001 ± 0.021; *Cox 2* = 1.017 ± 0.089; cox-$2^{-/-}$: *Cox 1* = 2.092 ± 0.078; *Cox 2* = 0.011 ± 0.0005).

<Figure 7 near here>

Discussion

Here we provide the evidence that the genetic deletion of Cox-2 from neutrophils promoted an increase in IL-1β and CXCL-1 release and neutrophil migration, in addition to a COX-1 overexpression in the inflamed tissue, resulting in the hyperalgesia intensification.

The synthesis of COX-2 is triggered mainly by pro-inflammatory stimuli, showing an essential role in PG's production [24]. Also, COX-2 inhibition is a relevant pharmacological tool in pain management [24]. In this context, neutrophils are the first cells migrating to the inflammatory site, showing relevant participation in the inflammatory progression and resolution through their production of cytokines and COX-2-derived eicosanoids [25]. In the present study, we demonstrated the importance of neutrophil-derived COX-2 in inflammatory hyperalgesia.

Our results showed, for both genders, higher CG-induced mechanical hyperalgesia in Cox-$2^{fl/fl:\ Mrp8cre+/-}$ mice when compared to Cox-$2^{fl/fl}$ mice. It is essential to mention that CG induces an acute and localized inflammation, which involves the participation of the resident and migratory cells (*e.g.*, macrophages and neutrophils), besides inflammatory mediators (*e.g.*, eicosanoid, cytokines, and chemokines) [26]. In addition, CG-induced hyperalgesia is Cox-dependent as evidenced by the fact that indomethacin, a nonspecific Cox inhibitor, inhibits this pro-nociceptive effect [27]. Also, worth mentioning that Cox-1 is described mainly as a constitutive isoform, regulating several physiological processes [28]. On the other hand, Cox-2 is defined as an inducible isoform, especially synthesizing prostanoids following an inflammatory stimulus [29]. Thus, the result mentioned above prompted us to question which factors are related to the more intense CG-induced hyperalgesia in Cox-$2^{fl/fl:\ Mrp8cre+/-}$ mice.

Consistent with what was already described [30], local CG injection evokes the production of pro-inflammatory cytokines. Nonetheless, interestingly we observed a significant upregulation of IL-1β and CXCL-1 in Cox-$2^{fl/fl:\ Mrp8cre+/-}$ mice compared with Cox-$2^{fl/fl}$. While CXCL-1 plays a crucial role in the chemoattraction of neutrophils cells during its initial phase [26, 31], IL-1β is one of the primary stimuli for Cox-2 expression during inflammation [32, 33]. This profile of exacerbation response was already described in Cox-$1^{-/-}$ or Cox-$2^{-/-}$ mice and isolated cells (*e.g.* fibroblasts) [34–36]. This exacerbated response includes enhanced eicosanoids and oxidative stress products [34–36]. Agreeing with our data, this exacerbated response in Cox-$2^{fl/fl:\ Mrp8cre+/-}$ mice extends to the increased release of cytokines in inflammatory hyperalgesia. Additionally, considering the higher IL-1β levels in the inflamed tissue of Cox-$2^{fl/fl:\ Mrp8cre+/-}$ mice, we investigated if IL-1β injection would trigger more intense hyperalgesia in this group. Based on this, we demonstrated that Cox-$2^{fl/fl:\ Mrp8cre+/-}$ mice showed long-lasting mechanical hyperalgesia after IL-1β injection when compared to Cox-$2^{fl/fl}$. IL-1β is an important CG-derived molecule and is closely associated with inflammatory hyperalgesia [26].

Considering the neutrophil as a key cell type to the acute inflammatory establishment [25], in the present study we quantified MPO after CG injection as a marker for its migration. Our data showed that Cox-$2^{fl/fl:\ Mrp8cre+/-}$ mice display higher levels of MPO when compared to Cox-$2^{fl/fl}$, which demonstrates that Cox-

$2^{fl/fl: Mrp8cre+/-}$ mice show an exacerbated neutrophil migration. Complementing the MPO analysis, in flux cytometry, we observed a significant increase in neutrophil cells at the peak of the acute CG-action, with no significant difference in macrophages and dendritic cells quantity. Indeed, neutrophils are predominant in the early stages of inflammation, usually preceding the recruitment of monocyte-derived macrophages[37]. Further, Corroborating this data, using a leukocyte-migration inhibitor (FC) [38], we showed the critical participation of neutrophil cells in CG-induced hyperalgesia for Cox-$2^{fl/fl: Mrp8cre+/-}$ mice. Our results demonstrated that pre-treatment with FC significantly reduced hyperalgesia in Cox-$2^{fl/fl}$ and Cox-$2^{fl/fl: Mrp8cre+/-}$ mice. It is well established that the neutrophil cells' influx into the inflammatory site is one of the main events related to acute inflammation [39]. It is also well described that neutrophils are the main leukocytes in the inflammatory site three hours after CG injection (acute phase) [40]. Moreover, during the initial phase of inflammation, there is increased Cox-2 expression in neutrophils, followed by PGE_2 production, which is one of the main responsible for the sensitization of the nociceptors, and consequently the hyperalgesia [41].

Based on our results regarding the different responses to inflammatory stimulation with CG in Cox-$2^{fl/fl}$ and Cox-$2^{fl/fl: Mrp8cre+/-}$ mice, we compare *Cox-1*, and *Cox-2* expression levels for both groups challenged with CG in the hind-paw. Additionally, to validate our knockout model we also evaluated *Cox-1*, and *Cox-2* expression levels in isolated neutrophils stimulated with lps. As expected, the COX-2 gene was upregulated during inflammatory stimuli in both, tissue and neutrophil cells Cox-$2^{fl/fl}$ with no significant changes in basal levels of *Cox-1* expression. In contrast, Cox-$2^{fl/fl: Mrp8cre+/-}$ mice showed a significant *Cox-1* upregulation, while *Cox-2* levels were almost undetectable by the method employed, in both tissue and isolated neutrophils. Indeed, the induction of *Cox-2* upregulation is standard in several pathological conditions and responses to inflammatory stimuli (*e.g.*, cytokines) [42]. On the other hand, interestingly, we demonstrated that the lack of COX-2 on neutrophil cells triggers a significant upregulation in Cox-*1* levels after inflammatory stimulation. Our data agree that, as shown previously [35, 36], Cox expression can be re-programmed to an alternate isoform in knockout mice [36] and cells [35]. In addition, literature data suggest a collaborative role of both Cox-1 and – 2 during inflammation [43]. Thus, we consider simplistic the view that only COX-2 can be induced and contribute to inflammatory processes.

Based on these results, we also evaluated the possible COX-1 involvement in CG-induced hyperalgesia in Cox-$2^{fl/fl: Mrp8cre+/-}$ mice using a specific COX-1 inhibitor (VS) [44]. Our data demonstrated that VS pre-treatment did not change the hyperalgesia intensity in Cox-$2^{fl/fl}$ mice. However, interestingly, we observed a reduction of the hyperalgesia in Cox-$2^{fl/fl: Mrp8cre+/-}$ mice with VS pre-treatment, attesting that, at least in part, Cox-1 may be mediating hyperalgesia in these mice. Indeed, no effect in hyperalgesia intensity after Cox-1 inhibition in Cox-$2^{fl/fl}$ group was not a surprise, given the well-established Cox-2 role in the inflammatory hyperalgesia [45]. However, the possible Cox-1 compensation in consequence of Cox-2 absence in neutrophils was perplexing. Thus, despite the well-established Cox-1 homeostatic functions and housekeeping activities, our results support previous studies demonstrating that Cox-1 could contribute to the inflammatory response [35, 43, 46].

In summary, our data suggest that the absence of Cox-2 in neutrophil cells exacerbates the hyperalgesia intensity and the inflammatory process induced by CG. It is plausible to hypothesize that a compensatory Cox-1 action mediates this effect. Finally, our data showed that this inflammatory exacerbation is associated with an increase in inflammatory mediators (IL-1β and CXCL-1) and neutrophil migration. Considering the abovementioned Cox-1 overexpression in Cox-2$^{fl/fl: Mrp8cre+/-}$ mice, it is plausible to use this model to study Cox-1 effects and downstream signaling. Understanding the role of Cox isoforms in inflammatory processes is essential to improving and developing new therapies targeted to its signaling pathways.

Abbreviations

COX - Cyclooxygenase

CG - Carrageenan

FC - Fucoidan

IL- Interleukin

i.p - Intraperitoneal

KO - knocked out

LPS - Lipopolysaccharide

MPO - Myeloperoxidase

NSAIDs - Non-Steroidal Anti-Inflammatory Drugs

PGs -Prostaglandins

TNF - Tumor Necrosis Factor

Veh - Vehicle

VS - Valeryl Salicylate

Declarations

Ethics Approval

All procedures carried out in this study were approved by the Animal Research Ethics Committee – University of Campinas (CEUA 4564-1/2017), that approved all treatments, procedures, and experimental protocols used in this study. Mice care, handling, and behavioral experiments followed the International

Association for the Study of Pain (IASP) guidelines for the use of animals in pain research. All efforts were made to ensure minimal mice use.

Consent to participate and Consent to publish

Not applicable.

Material and/or Code availability

Not applicable.

Competing Interests

The authors declare no competing interests.

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Author contribution

This study was conceived and designed by Carlos Amilcar Parada, Marco Ramirez Vinolo and Nathalia Santos Carvalho. Material preparation and data collection were performed by Nathalia Santos Carvalho, Julia Borges Paes Lemes, Marco Pagliusi Jr, Ana Carolina Machado, Kauê Franco Malange, Laís Passariello Pral, José Luís Fachi, Catarine Nishijima Massucato and Gilson Gonçalves dos Santos. Statistical analyzes were performed by Nathalia Santos Carvalho, Claudia Herrera Tambeli and Cesar Sartori. The first draft of the manuscript was written by Nathalia Santos Carvalho, Marco Pagliusi Jr, and Ana Carolina Machado, which was critically reviewed and revised by Carlos Amilcar Parada, Marco Ramirez Vinolo, Gilson Gonçalves dos Santos, Claudia Herrera Tambeli and Cesar Sartori. All authors read and approved the final manuscript. All authors agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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Tables

Table 1 is available in the Supplementary Files section.

Figures

Figure 1

Hyperalgesia intensity after CG paw injection. (A) Shows the intensity of hyperalgesia at 1h, 2h, 3h, 4h, 5h, 6h, and 24h after carrageenan (CG) injection for both groups, Cox-$2^{fl/fl}$ and Cox-$2^{fl/fl: Mrp8cre+/-}$. Note the highlighted time point (3h) showing the peak of hyperalgesia. **(B)** Shows the area under the curve. **(C) S**hows the intensity of hyperalgesia 3h after CG injection for both genders of both groups (N=6-8 per group; $*p \leq 0.05; **p \leq 0.01; ***p \leq 0.001$).

Figure 2

Profile of pro-inflammatory cytokines released in the inflamed tissue of these mice. (A). **(B), (C)** and **(D)** shows the level of Cinc-1, IL-1β, TNFα, and IL-10, respectively, released after 3h CG stimulus in paw for both groups (N=6 per group). The symbol (*) indicates a statistical difference when compared to intra-groups. The symbol ($^{\#}$) indicates a statistical difference comparing between groups. (N=5-6 per group; $*p \leq 0.05$; $**p \leq 0.01$ ***, $^{\#\#\#}p \leq 0.001$).

Figure 3

Long-lasting hyperalgesia following IL-1β injection. Shows the intensity of hyperalgesia 20 min, 40 min, 60 min, 90 min, 120 min, 150 min, and 180 min after IL-1β injection for both groups, Cox-$2^{fl/fl}$ and Cox-$2^{fl/fl: Mrp8cre+/-}$ **B)** shows the area under the curve. (N=5-6 per group; $***p \leq 0.001$).

Figure 4

Quantification of cell migration after CG injection. (A) Shows the myeloperoxidase (MPO) quantification in the hind paw after 3h CG or Veh injection. **(B) s**hows the representative flow cytometry dot plots of Ly6G+CD11b+neutrophil, CD11b+F4/80+ macrophage, and CD11b+CD11c+ dendritic cells on CD45+ compartment, from peritoneal washing after CG or Veh *i.p.* injections. **(C)**, **(D)** and **(E)** Show the percentage of neutrophils, macrophage, and dendritic cells, respectively, in peritoneal washing after CG or Veh *i.p.* injections for both groups. The symbol (*) indicates a statistical difference when compared to intra-groups. The symbol ($^{\#}$) indicates a statistical difference comparing between groups. ($**p \leq 0.01$; $***p \leq 0.001$; $^{\#}p \leq 0.05$ and $^{\#\#\#}p \leq 0.001$).

Figure 5

Hyperalgesia intensity after CG + FC injection. Shows the intensity of hyperalgesia 3h after carrageenan (CG) and fucoidan (FC). (N=5 per group; ***$p \leq 0.001$).

Figure 6

***Cox-1* and *cox-2* expression in the hind pawn tissue and hyperalgesia ntensity after CG + VS injection. A)** **S**hows *cox-1* and *cox-2* mRNA expression (fold change using a vehicle (Veh) as standard) in the hind paw tissue for both groups after 3h CG or Veh injection. **B)** Shows the intensity of hyperalgesia 3h after CG and valeryl salicylate (VS) or CG and Veh injection for both groups. (N=5 per group; *$p \leq 0.05$; **$p \leq 0.01$, ***$p \leq 0.001$, ns=no significant).

Figure 7

***Cox-1* and *cox-2* expression in neutrophils stimulated by LPS *in vitro*. (A)** Shows the experimental graphical design for this experiment. **(B)** Shows *Cox-1* an C*ox-2* mRNA expression for both groups (N=6 per group; ***p≤0.001).

Supplementary Files

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- Table1.jpg