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Original article

Electrochemical evidence of nitrate release from the nitrooxy compound 4-((nitrooxy) methyl)-3-nitrobenzoic acid and its antinociceptive and anti-inflammatory activities in mice

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ABSTRACT

Considering the many biological activities of nitric oxide (NO), some lines of research focused on the modulation of these activities through the provision of this mediator by designing and synthesizing compounds coupled with an NO donor group. Thus, the objectives of the present study were to carry out an electrochemical investigation of the nitrooxy compound 4-((nitrooxy) methyl)-3-nitrobenzoic acid (**1**) and evaluate its activities and putative mechanisms in experimental models of pain and inflammation. Voltammetric studies performed in aprotic medium (mimetic of membranes) showed important electrochemical reduction mechanisms: nitroaromatic reduction, self-protonation, and finally reductive elimination, which leads to nitrate release. Systemic administration of the nitrooxy compound **(1)** inhibited the nociceptive response induced by heat and the tactile hypersensitivity and paw edema induced by carrageenan in mice. The activities in the models of inflammatory pain and edema were associated with reduced neutrophil recruitment and production of inflammatory cytokines, such as interleukin (IL)-1β, IL-6, tumor necrosis factor-α and CXCL-1, and increased production of IL-10. Concluding, electrochemical analysis revealed unequivocally that electron transfer at the nitro group of the nitrooxy compound (**1**) results in the cleavage of the organic nitrate, potentially resulting in the generation of NO. This electrochemical mechanism may be compared to a biochemical electron-transfer mediated nitrate release that, by appropriate *in vivo* bioreduction (enzymatic or not) would lead to NO production. Compound (**1**) exhibits activities in models of inflammatory pain and edema that may be due to reduced recruitment of neutrophils and production of inflammatory cytokines and increased production of IL-10. These results reinforce the interest in the investigation of NO donor compounds as candidates for analgesic and anti-inflammatory drugs.

1. Introduction

Nitric oxide (NO) is a simple molecule that exerts several physiological functions. Endogenous NO is synthesized from *L*-arginine by NO synthase (NOS) enzymes. The main NO-mediated signaling occurs through the activation of the cytosolic enzyme guanylyl cyclase, resulting in the formation of cyclic GMP (cGMP) which regulates several important intracellular pathways. NO is a mediator present in almost all cells, exerting different functions such as vascular tone control, neurotransmission, induction of gene expression of transcription factors and cellular apoptosis [1,2]. NO may also exert important signals in the regulation of the immune response such as inhibition of NF-κB [3],

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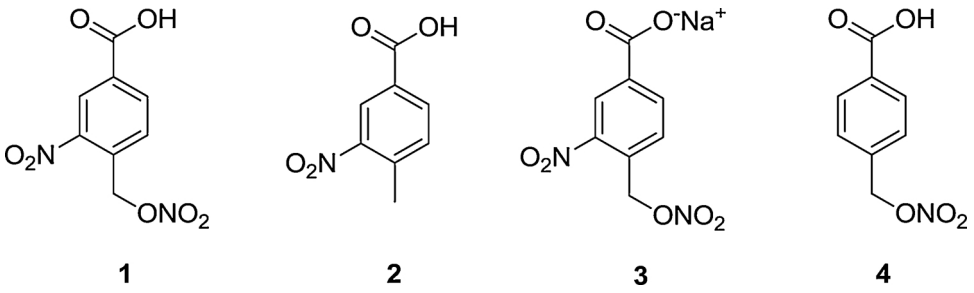


Fig. 1. Chemical structures of the compounds (1-4).

resulting in decreased synthesis of many inflammatory mediators.

Considering the biological activities of NO, some lines of research focused on the modulation of these activities, through the provision of this mediator, by designing and synthesizing compounds coupled with an NO donor group. Organic nitrates, synthesized by choice of the appropriate chemical spacer arm carrying the nitric ester, have been obtained, aiming to achieve NO-releasing compounds [4]. The NO release from organic nitrates requires either enzymatic or nonenzymatic bioactivation, where a three-electron reduction thiol-dependent is involved [5–9]. NO is produced from L-arginine in the vascular endothelium by NOS [10] and from nitrite/nitrate in the erythrocyte by deoxyhemoglobin [11] or in the stomach or tongue by sequential reduction of nitrate [12–16]. Nitrate and nitrite are physiologically recycled in blood and tissue to form NO and other bioactive nitrogen oxides. Thus, they should be viewed as storage pools for NO-like bioactivity, thereby complementing the NO synthase-dependent pathway [17].

There is a progressive overlap of interests between electrochemistry and other areas of research, mainly biologically based ones [18]. Electrochemical methods are considered useful tools for simulations of biological (i.e. metabolic) redox reactions, which represent preliminary and essential pathways in the oxidative stress [19,20]. The cathodic reduction of nitro compounds is generally very well understood [21,22]. The interest in this class of compounds is mainly due to their medicinal uses [23]. Their electrochemical behaviors depend strongly on their chemical structures and experimental conditions: the solvent, supporting electrolyte, pH values and nature of the electrode [21,24,25]. Concerning organic nitrates, electrochemical studies have shown that adequately located organic nitrates can release nitrates, after reduction through reductive elimination [26].

Thus, the objectives of the present study were to carry out an electrochemical investigation of the novel 4-((nitrooxy) methyl)-3-nitrobenzoic acid (**1**, Fig. 1), a NO donor, and evaluate its effects in experimental models of pain and inflammation. In electrochemical terms, 4-((nitrooxy) methyl)-3-nitrobenzoic acid (**1**) has two electroactive functions and their mutual influences were also explored. Thus, the 4-methyl-3-nitrobenzoic acid (**2**), the sodium salt of **1** (**3**) and the 4-((nitrooxy)methyl)benzoic acid (**4**) were also prepared and investigated for the sake of electrochemical mechanism clarification (Fig. 1).

2. Material and methods

2.1. Chemistry

All melting points were determined on a Microquímica MQAPF 301 apparatus. The IR spectra were recorded on a PerkinElmer Spectrum One infrared spectrometer and absorptions are reported as wave numbers (cm^{-1}). The NMR spectra were recorded on a Bruker AVANCE DRX200 or Bruker AVANCE DRX400 instrument, using tetramethylsilane as the internal standard. Chemical shifts are given in δ (ppm) scale and J values are given in Hz. All reagents of analytical grade were obtained from commercial suppliers and used without further purification.

2.2. Synthesis of 4-((nitrooxy)methyl)-3-nitrobenzoic acid (1)

In a round-bottom flask containing concentrated sulfuric acid (2.0 mL), 4-(hydroxymethyl)-3-nitrobenzoic acid (0.2 g; 1.02 mmol) was added slowly to the solution in an ice bath under stirring. Fuming nitric acid (1.0 mL) was then added dropwise. The ice bath was removed and the reaction was kept under magnetic stirring for 2 h. After the reaction period, crushed ice was added to the mixture and the precipitate was filtered off and washed with cold water, to yield the desired nitrooxy compound (**1**) as a pale solid, 67 % yield. m.p. 108.8–109.6 °C; ^{1}H NMR (200 MHz, acetone-*d6*) δ 8.71 (s, 1 H), 8.41 (d, 1H, J =8.0 Hz), 7.91 (d, 1H, J =8.0 Hz), 6.08 (s, 2 H); ^{13}C NMR (50 MHz, acetone-*d6*) δ 165.30 (C = O), 148.71 (C), 135.42 (CH), 134.18 (C), 133.29 (C), 130.98 (CH), 126.88 (CH), 71.46 (CH_2).

2.3. Synthesis of sodium 3-nitro-4-((nitrooxy)methyl)benzoate (3)

Sodium carbonate (0.005 g, 0.04 mmol) and ethanol (2 mL) were added to a round-bottom flask containing the nitrooxy compound (**1**) (0.02 g, 0.08 mmol). The mixture was stirred at room temperature for 2 h, and then, the solvent was removed under reduced pressure to yield the sodium salt (**3**) as a brown-yellow solid, 99 % yield. m.p. 236−240 °C; ^{1}H NMR (400 MHz, methanol-*d4*) δ 8.60 (s, 1 H), 8.19 (d, 1H, J =8.0 Hz), 7.59 (d, 1H, J =8.0 Hz), 5.85 (s, 2 H); ^{13}C NMR (100 MHz, methanol-*d4*) δ 171.53 (C = O), 141.70 (C), 135.29 (CH), 131.32 (C), 130.58 (CH), 126.80 (CH), 71.98 (CH_2).

2.4. Synthesis of 4-methyl-3-nitrobenzoic acid (2) and 4-(nitrooxy) methyl)benzoic acid (4)

The 4-methyl-3-nitrobenzoic acid (**2**) [27] and 4-(nitrooxy)methyl) benzoic acid (**4**) [28] were synthesized according to the published procedures.

2.5. Electrochemical studies

Extra-dry *N*,*N*-dimethylformamide (DMF; 99.8 %) was acquired from Acros Organics (USA), tetrabutylammonium hexafluorophosphate ($TBAPF_6$), tetrabutylammonium tetrafluoroborate ($TBABF_4$) and the other chemicals from Sigma-Aldrich (USA). $TBAPF_6$ was purified by recrystallization from absolute ethanol (×3) and dried at reduced pressure, at 60 °C. All the reagents were analytical grade. All the solutions were prepared using ultrapure water (18.2 MΩ cm) from Milli-Q (Millipore Inc.).

The electrochemical studies, mainly cyclic voltammetry, of 4-((nitrooxy)methyl)-3-nitrobenzoic acid (**1**) and its sodium salt (**3**), as well as of the 4-methyl-3-nitrobenzoic acid (**2**) and 4-((nitrooxy) methyl)-derivative (**4**) were performed on an Autolab PGSTAT-30

potentiostat (Echo Chemie, Utrecht, the Netherlands) coupled to a PC microcomputer, using GPES 4.9 software. The working electrode was a glassy carbon electrode (GCE) (d =3 mm), the counter electrode, a Pt wire, and the reference electrode Ag|AgCl|Cl^- (saturated). All electrodes were contained in a one-compartment Pyrex glass electrochemical cell with a volumetric capacity of 5 mL. GCE was cleaned up by polishing with alumina on a polishing felt (BAS polishing kit). Electrochemical reduction was performed in aprotic media, (DMF + $TBAPF_6$ 0.1 mol L^{-1} or DMF + $TBABF_4$ 0.1 mol L^{-1}), at room temperature (25 ± 2 °C). Each compound (1×10^{-3} mol L^{-1}) was added to the supporting electrolyte, and the solution was deoxygenated with argon, before the CV measurements. The most representative potential range was from 0 to -2.2 V *vs*. Ag|AgCl|Cl^- (sat.). In CV experiments, the scan rate varied from 10 to 500 mV s-. Electrochemical reduction was performed in aprotic media (DMF + $TBAPF_6$ 0.1 mol L^{-1}), at room temperature (25 ± 2 °C). Each compound (1×10^{-3} mol L^{-1}) was added to the supporting electrolyte, and the solution was deoxygenated with argon, before the CV measurements. All the CVs were displayed at scan rates of 100 mV s^{-1}

2.6. Biological assays

2.6.1. Animals

Female Swiss mice weighing 25−30 g were used. The animals were acclimatized to a room with a 12 h light-dark cycle for at least three days before the experiment. The study was approved by the Ethics Committee on Animal Experimentation of the Federal University of Minas Gerais (Protocol 23/2016) and carried out according to the ethical guidelines for investigation of experimental pain in conscious animals [29].

2.6.2. Drugs

Dexamethasone 21-phosphate disodium salt, λ-carrageenan (Sigma-Aldrich, USA), polyethylene glycol 400 (PEG 400; Synth, Brazil), phenobarbital, dipyrone (Sanofi Aventis, Brazil) and ketamine and xylazine (Ceva Santé Animale, Brazil) were used. Suspension of the nitrooxy compound **(1)** was prepared in saline (NaCl 0.9 %; pH 6.8) containing 40 % (v/v) PEG 400 and administered via the intraperitoneal (i.p.) route in a volume of 4 mL/kg. Solutions of dexamethasone, phenobarbital, dipyrone, ketamine and xylazine were prepared in saline. These solutions were administered per os (*p.o.*) in a volume of 10 ml/kg or i.p. in a volume of 4 mL/kg. Carrageenan suspension (2%) was prepared in saline and administered via the intraplantar (i.pl.) route. All solutions and suspensions were prepared immediately before each experiment.

2.6.3. Evaluation of the nociceptive response induced by heat

A hot plate apparatus (Model EFF 361, Insight, Brazil) was used to evaluate the nociceptive response induced by heat, as described by Dutra et al. [30]. Each animal was placed on the hot plate (50 °C) 30 min after administration of the nitrooxy compound (**1**) (50, 100 or 150 mg/kg, i.p), dipyrone (500 mg/kg, *p.o.*) or vehicle (PEG 400 40 %, 4 mL/kg, i.p.). The latency (s) to lick one of the hind paws or jump off the plate was determined. The animal was removed from the hot plate immediately after the response. The cut-off time was 50 s to avoid tissue damage.

2.6.4. Evaluation of the tactile hypersensitivity induced by carrageenan

Tactile hypersensitivity was measured by using an electronic von Frey apparatus (Model EFF 301, Insight, Brazil) as described by Brito et al. [31]. The mice were kept individually in acrylic cages whose floor was a metal grid. The animals were habituated to the experimental apparatus daily, approximately 60 min a day, for two days before the experiments. A hand-held force transducer, fitted with a polypropylene tip (0.5 mm^2), was gradually pressed onto the plantar surface of the right hind paw. The test consisted of evoking a reflex of hind paw flexion. The paw withdrawal threshold (PWT) was determined by averaging five measurements. On the experimental day, baseline PWT of each animal was determined. After that, the animals were divided into the experimental groups in such a way that the mean PWT of the different groups were similar. On the experimental day, carrageenan (400 μg, 20 μL) was injected 30 min after i.p. administration of the nitrooxy compound (**1**) (50, 100 or 150 mg/kg), dexamethasone (2 mg/kg) or vehicle (PEG 400 40 %, 4 mL/kg). The PWT of each animal was again measured at 1, 3, 5 and 7 h after carrageenan injection. The results were expressed as the absolute PWT (g).

2.6.5. Evaluation of the motor activity

The motor activity of the animals was evaluated on a rota-rod apparatus as described by Morais et al. [32]. Two days before the evaluation, the animals were trained on the apparatus. To evaluate the motor activity, the animals were placed on the rota-rod (14 rpm) and the time (s) they spent on it was determined. The cut-off time was 120 s. After determination of the baseline values, the animals were treated with the nitrooxy compound (**1**) (150 mg/kg, i.p.), phenobarbital (50 mg/kg, *p.o.*) or vehicle (PEG 400 40 %, 4 mL/kg, i.p.) and tested again in the apparatus 0.5, 1, 3, 5 and 7 h later.

2.6.6. Evaluation of the paw edema induced by carrageenan

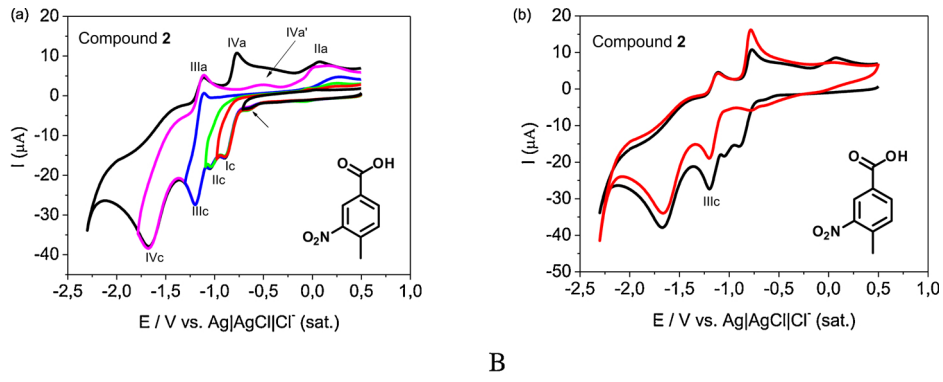
Paw edema was measured with a plethysmometer (Model 7140, Ugo Basile, Italy) as described by Godin et al. [33]. The basal volume of the right hind paw was measured before administration of any drug. Next, the animals were divided into the experimental groups in such a way that the mean paw volumes of the different groups were similar. On the experimental day, carrageenan (400 μg, 20 μL) was injected 30 min after i.p. administration of the nitrooxy compound (**1**) (50, 100 or 150 mg/kg), dexamethasone (2 mg/kg) or vehicle (PEG 400 40 %, 4 mL/kg). The paw volume of each animal was again measured at 1, 3, 5 and 7 h after injection of the inflammatory stimulus. The results were expressed as the paw volume change (μL) in relation to the basal values.

2.6.7. Evaluation of tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, IL-10 and chemokine (C-X -C motif) ligand 1 (CXCL-1) production induced by carrageenan

Carrageenan (400 μg, 20 μL) was injected via the i.pl. route 30 min after i.p. administration of the nitrooxy compound (**1**) (150 mg/kg) or vehicle (PEG 400 40 %, 4 mL/kg). TNF-α, IL-1β, IL-6, IL-10 and CXCL-1 concentrations were measured in the ipsilateral footpad tissue using ELISA assays, following the instructions supplied by manufacturer (DuoSet kits, R&D Systems, USA). The animals were anesthetized with a mixture of ketamine and xylazine (100/10 mg/kg, i.p.) and euthanized by cervical dislocation 5 h later and the paw tissue was removed. The tissues were weighed and homogenized in phosphate buffered saline containing Tween-20 (0.05 %), phenylmethylsulphonyl fluoride (0.1 mM), benzamethonium chloride (0.1 mM), EDTA (10 mM), aprotinin A (2 μg/mL) and bovine serum albumin (0.5 %), followed by centrifugation (10,000 rpm) for 15 min at 4 °C. The supernatant samples were stored at −70 °C until analysis of cytokines concentrations. All samples were assayed in duplicate and the results were expressed as pg/100 mg of tissue.

2.6.8. Evaluation of the plasma concentration of nitrite

Five hours after the i.p. administration of the nitrooxy compound (**1**) (150 mg/kg) or vehicle (PEG 400 40 %, 4 mL/kg), each animal was anesthetized with a mixture of ketamine and xylazine. After decapitation, a blood sample was collected from each animal in a heparinized



A B

Fig. 2. Cyclic voltammograms of 4-methyl-3-nitrobenzoic acid (**2**) (1 mmol L^{-1}) in DMF + $TBABF_4$ 0.1 mol L^{-1}, GCE. $\nu = 0.100$ V s^{-1}. (A) Several reversal potentials. (B) Consecutive scans, with remark on peak IIIc.

tube and immediately centrifuged at 2000 rpm for 20 min at 4 °C. Then, the plasma samples were transferred to plastic tubes and stored at −70 °C until analysis. Plasma samples were analysed using a procedure based on the Griess reaction [34]. Briefly, 60 μL of Griess reagent (1% sulfanilamide and 0.1 % napthylethylenediamine in 2.5 % phosphoric acid) were added to 60 μL of plasma samples. After 10 min, the absorbance of the samples were measured at 540 nm. A calibration curve ranging from 5 to 240 μM of sodium nitrite was prepared in BSA and measured simultaneously to quantify nitrite in the samples. The results were expressed in μM.

2.6.9. Evaluation of the myeloperoxidase (MPO) activity

The pellet samples collected after the removal of the supernatants were frozen and thawed three times in liquid nitrogen. Upon thawing, the samples were centrifuged (10,000 rpm) for 15 min at 4 °C and 25 μL of the supernatant were used for the MPO assay as previously described [35]. The enzymatic reaction was assessed by adding 25 μL of 1.6 mM 3, 3′, 5,5′-tetramethylbenzidine substrate prepared in dimethyl sulfoxide. The mixture was incubated for 5 min at 37 °C. Then, 100 μL of 0.002 % hydrogen peroxide were added, followed by incubation for 5 min at 37 °C. After 10 min, the reaction was stopped by adding 100 μL of 1 M sulphuric acid. The MPO activity was expressed as optical density (OD)/100 mg of tissue and absorbance was measured at 450 nm.

2.6.10. Statistical analysis

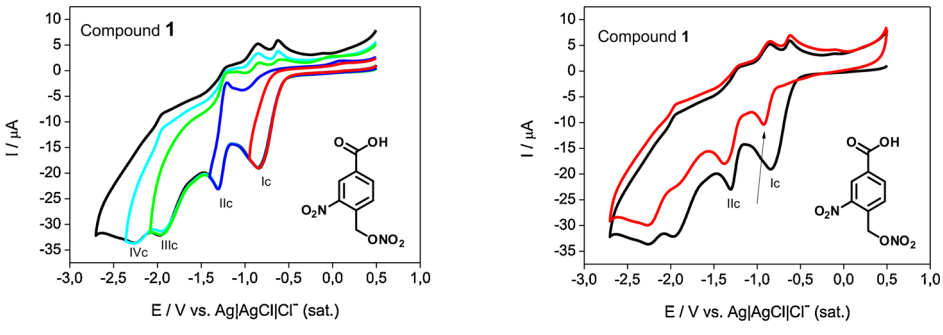
All data were presented as the mean ± standard error of the mean. Both temporal changes and areas under the curves (AUC) were shown. Differences were evaluated by using one-way ANOVA followed by Newman-Keuls *post-hoc* test or *t* test. Two-way ANOVA followed by Bonferroni post hoc test was used to analyse PWT and paw volumes differences. A p value < 0.05 was considered significant. Statistical analysis was conducted using GraphPrism 5.0 for Windows software.

3. Results and discussion

3.1. Electrochemistry

As the nitrooxy compound (**1**) has two redox functionalities (nitro and nitrooxy), both of them influenced by the presence of the carboxylic acid, four compounds (**1**-**4**) were investigated for a better understanding of the electrochemical outcome.

Fig. 2 A and B display the cyclic voltammograms (CV) of compound (**2**), the 4-methyl-3-nitrobenzoic acid, obtained in *N*,*N*-dimethylformamide (DMF), using $TBABF_4$ as a supporting electrolyte. Four reduction processes were observed – peaks Ic (-0.874 V) and IIc (-1.031 V) (shoulder) have irreversible characteristic, while peak IIIc (-1.194 V) has an anodic counterpart (-1.120 V, $\Delta Ep = 0.074$ V), being reversible, and is followed by peak IVc (EpIVc = -1.672 V) (Fig. 2A). The anodic counterpart presents additional anodic waves (Fig. 2A). This reduction process can be understood in the context of the well-known mechanism of self-protonation [36], previously reported for 4-nitrobenzoic acid [37], lapachol [38], and 4-nitrophenylacetic acid [39]. The reduction at Ic, at more positive potential (E_{pc} = -0.874 V), is related to the full reduction of 1/5 of the original compound to the corresponding hydroxylamine, as depicted in Eq. 1, and followed by the reversible reduction of 4/5 of the conjugate base ($NO_2CH_3PhCOO^-$), at EpIIIc



A B

Fig. 3. Cyclic voltammograms of the nitrooxy compound (**1**) (1 mmol L^{-1}) in DMF + $TBAPF_6$ 0.1 mol L^{-1}, GCE. $\nu = 0.100$ V s^{-1}. (A) Several reversal potentials. Ic is a composed peak. (B) Consecutive scans, with an arrow on peak Ic, at the second scan and remark on peak IIc, both important for the electrodic mechanism elucidation.

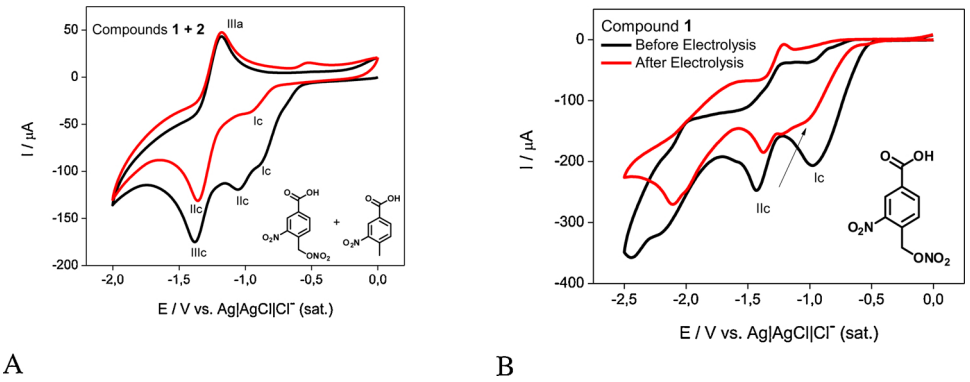


Fig. 4. Cyclic voltammograms in DMF + $TBAPF_6$ 0.1 mol L^{-1}, GCE. $\nu = 0.100$ V s^{-1}. (A) nitrooxy compound (**1**) (second scan, in red) (1 mmol L^{-1}) and compound (**2**) (in black) (1 mmol L^{-1}), Similarity between peak IIc of compound (**1**) and peak IIIc of compound (**2**). (B) Electrolysis of compound (**2**), held at *E*app. = -0.75 V. In black (before electrolysis) and in red (after electrolysis), with the arrow showing the remaining peak.

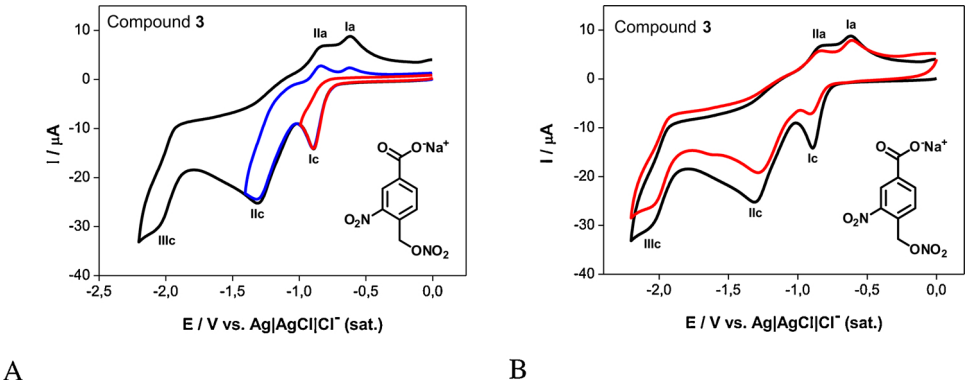


Fig. 5. Cyclic voltammograms of compound (**3**) (1 mmol L^{-1}), in DMF + $TBAPF_6$ 0.1 mol L^{-1}, GCE. $\nu = 0.100$ V s^{-1}. (A) Several reversal potentials. (B) Consecutive scans, with remark on peak IIIc.

(-1.194 V) and *E*pIIIa (-1.120 V), as depicted in Eq. 2 [37,39]. For the sake of simplicity, $NO_2CH_3PhCOOH$ represents compound (**2**) in Eq. 1 and $NO_2CH_3PhCOO^-$ represents the conjugate base of compound (**2**) in Eq. 2.

As recently reported for a nitrated phenylacetic acid [39], the intermediate shoulder (IIc) was assigned to the reduction of a hydrogen-bonding complex between the original carboxylic acid ($NO_2CH_3PhCOOH$) and its base ($NO_2CH_3PhCOO^-$), through a second self-protonation mechanism (Eq. 3). The last wave represents a further reduction of the carboxylate's nitroradical anion. As a characteristic of the self-protonation, in the second successive scan, the two first waves disappear, once the reaction is very fast and, at the reaction layer, only the carboxylate remains (Fig. 2B).

$$NO_2CH_3PhCOOH + 4/5e^- \rightarrow 4/5\ NO_2CH_3PhCOO^- + 1/5\ NHOHCH_3PhCOOH + H_2O \quad (1)$$

$$NO_2CH_3PhCOO^- + 4e^- + 4NHOHCH_3PhCOOH \rightarrow 5NHOHCH_3PhCOO^- + H_2O \quad (2)$$

$$[NO_2CH_3PhCOO^- \cdot\cdot H^+ \cdot\cdot {}^-OOCCH_3PhNO_2] + 4/5e^- \rightarrow 9/5\ NO_2CH_3PhCOO^- + 1/5\ NHOHCH_3PhCOOH + 2H_2O \quad (3)$$

These experiments were held as a reference to explain the voltammetric features of the hybrid nitro-nitrooxyderivative (**1**). For the sake of simplicity, the nitrooxy compound (**1**) is represented in the equations that follow by $NO_2(CH_2ONO_2)PhCOOH$, where -ONO_2 represents the nitrate group. As displayed in Fig. 3 A, the CV of the nitrooxy compound (**1**), as compared to the standard compound (**2**), is represented by a broad peak, assigned as Ic (*E*pIc = -0.848 V), followed by IIc (*E*pIIc = 1.302 V), with a quasi-reversible nature, as shown by the presence of the anodic peak IIa (*E*pIIa = -1.212 V) and relative to the reduction of the product from the self-protonation (Eq. 4), resulting in $NO_2(CH_2ONO_2)PhCOO^-$ (Eq. 4), plus the full reduction of the nitro group to the corresponding hydroxylamine ($NHOH(CH_2ONO_2)PhCOOH$). An additional electron transfer would result in the cleavage of the bond C-ONO_2, releasing the nitrate, by a process known as a dissociative electron transfer (Fig. 3 A–B) (Eq. 5). The presence of both processes can be suggested by comparing Fig. 3B vs. 2 B, where, on successive scans, one peak at *E*p ~-0.85 V was still evidenced (peak related to the C-ONO_2 cleavage).

Both processes would result in the conjugate base $NO_2CH_3PhCOO^-$ (Eqs. 5 and 6), already shown in Eq. 1.

$$NO_2(CH_2ONO_2)PhCOOH + 4/5e^- \rightarrow 4/5\ NO_2(CH_2ONO_2)PhCOO^- + 1/5\ NHOH(CH_2ONO_2)PhCOOH + H_2O \quad (4)$$

$$NO_2(CH_2ONO_2)PhCOO^- + e^- \rightarrow NO_2CH_2^{\bullet}PhCOO^- + {}^-ONO_2^{\bullet} \quad (5)$$

$$NO_2CH_2^{\bullet}PhCOO^- + e^- + H^+ \rightarrow NO_2CH_3PhCOO^- \quad (6)$$

Comparison of the CV of compounds (**2**) and (**1**), added together in the electrochemical cell, indicates, by the similarity of compound (**2**) *E*pIIc and compound (**1**) *E*pIIIc that the conjugate base of both compounds are the same (Fig. 4A). In a clearer view, voltammetric studies showed that the reductive elimination occurs, after nitroaromatic reduction. The $^-ONO_2$, instead of NO_2^-, release leads to electrogenerated methylene carbanion, easily protonated, furnishing at the reaction layer,

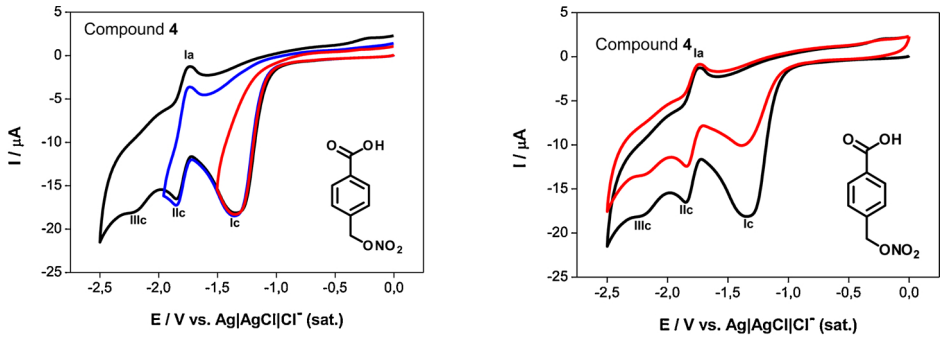


Fig. 6. Cyclic voltammograms of compound (**4**) (1 mmol L^{-1}), in DMF + $TBAPF_6$ 0.1 mol L^{-1}, GCE. $\nu = 0.100$ V s^{-1}. (A) Several reversal potentials. (B) Consecutive scans.

Table 1
Electrochemical parameters for the investigated compounds.

Comp.	*Ep*Ic (V)	*Ep*IIc (V)	*Ep*IIIc (V)	*Ep*IVc (V)	*Ep*Ia (V)	*Ep*IIa (V)	*Ep*IIIa (V)	*Ep*IVa (V)	*Ep*IVá (V)
1	−0.859	−1.310	−1.865	–	−0.626	−1.215	−0.838	–	–
2	−0.874	−1.031	−1.194	−1,672	–	−0.290	−1.120	−0.777	−0.512
3	−0.890	−1.302	−2.064	–	−0.620	−0.850	–	–	–
4	−1.319	−1.849	−2.183	–	−1.740	–	–	–	–

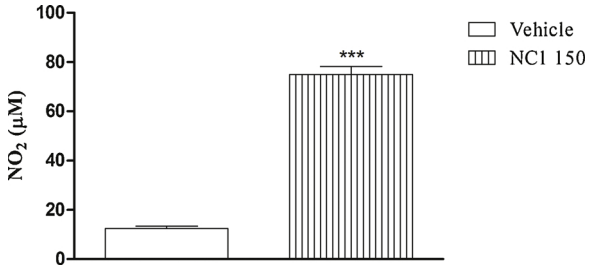


Fig. 7. Plasma concentrations of nitrite 5 h after i.p. administration of the nitrooxy compound (**1**) (NC1; 150 mg/kg) or vehicle (PEG 400 40 %, 4 mL/kg). *** significantly different from vehicle ($p < 0.001$; *t* test). n = 7.

the conjugate base of the nitrooxy compound (**1**) ($NO_2CH_3PhCOO^-$; Eq. 6), identical to the one obtained from compound (**2**) (Eq. 1). Electrolysis, followed by CV, was held, first, at the foot of the first wave (*E*app = -0.75 V) to prove the combined nature of the first wave (Fig. 4B). At this potential, the fast process of self-protonation occurred (Eq. 4).

Additional experiments with the sodium salt of compound (**1**) (carboxylate (**3**)) prove the mechanism (Fig. 5), once, as predicted, the self-protonation (Eq. 4) is excluded, with the reductive elimination (Eq. 5) being kept, as shown by the presence of a narrower wave Ic, in the CV of compound (**3**).

There is a precedent for the last reaction, reported for methylnitrooxy derivatives of quinones [26]. Despite the difference on the electrophore (quinone vs. nitroaromatic), the comparison is feasible, once both groups are reduced by one electron, generating stable anion radicals.

In sequence, two additional cathodic waves (IIIc, IVc), at -1.998 V and -2.259 V appear and are related to further reduction of the nitroanion radical (Fig. 3).

The cleavage of the C-leaving group bond, in this case, the nitrate, through a dissociative electron transfer at the potential of the first broad peak, would be expected to depend on the reduction potential of the reversible system, the strength of the C—X bond and the properties of X as a leaving group [40].

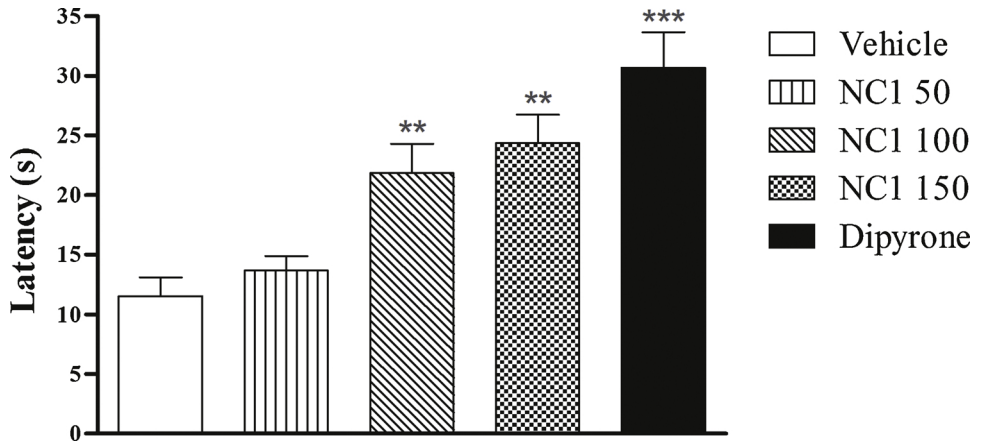


Fig. 8. Effect of the previous (30 min) administration of the nitrooxy compound (1) (NC1; 50, 100 or 150 mg/kg, i.p.), dipyrone (500 mg/kg, *p.o.*) or vehicle (PEG 400 40 %, 4 mL/kg, i.p.) on the nociceptive response induced by heat. ** and *** significantly different from vehicle ($p < 0.01$ and $p < 0.001$, respectively). One-way ANOVA followed by Newman-Keuls test. n = 6.

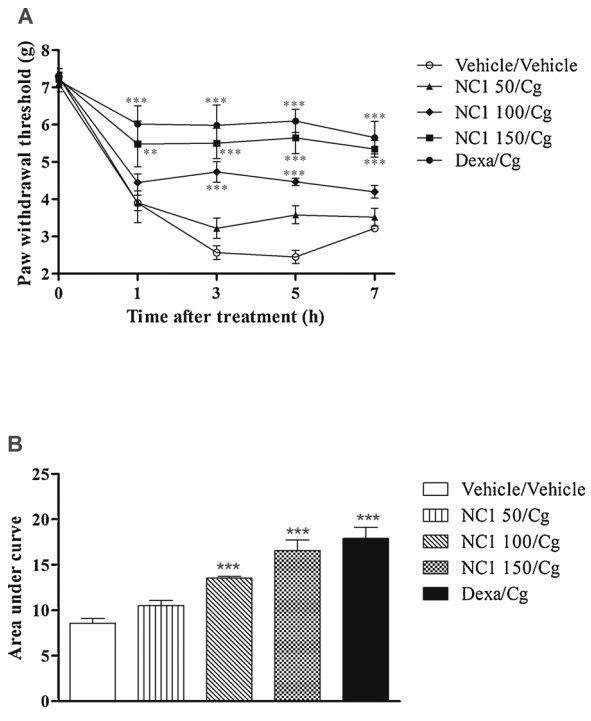


Fig. 9. Effect of the previous (30 min) i.p. administration of the nitrooxy compound (**1**) (NC1; 50, 100 or 150 mg/kg), dexamethasone (Dexa; 2 mg/kg) or vehicle (PEG 400 40 %, 4 mL/kg) on the tactile hypersensitivity induced by i.pl. injection of carrageenan (Cg; 400 μg). A represents the temporal course and B represents the AUC. ** and *** significantly different from vehicle ($p < 0.01$ and $p < 0.001$, respectively). (A) Two-way ANOVA followed by Bonferroni test and (B) one-way ANOVA followed by Newman-Keuls test. n = 6.

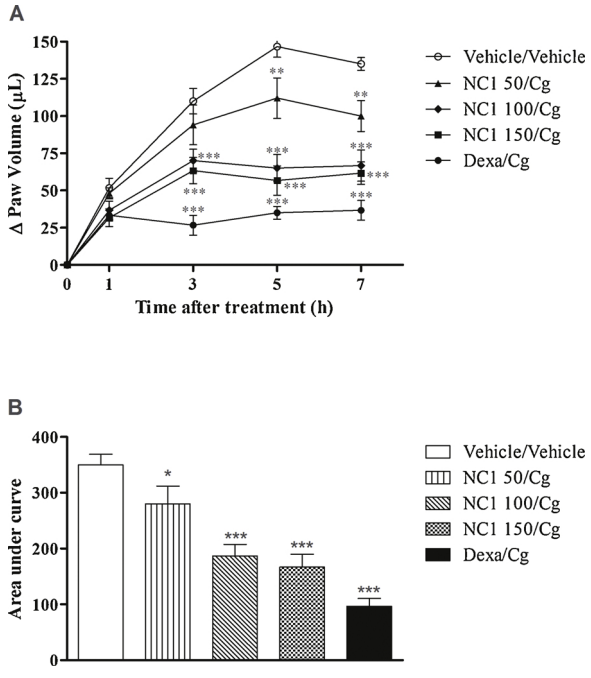


Fig. 10. Effect of the previous (30 min) i.p. administration of the nitrooxy compound (**1**) (NC1; 50, 100 or 150 mg/kg), dexamethasone (Dexa; 2 mg/kg) or vehicle (PEG 400 40 %, 4 mL/kg) on the paw edema induced by the i.pl. injection of carrageenan (Cg; 400 μg). A represents the temporal course and B represents the AUC. *, ** and *** significantly different from vehicle ($p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively). (A) Two-way ANOVA followed by Bonferroni test and (B) one-way ANOVA followed by Newman-Keuls test. n = 6.

Finally and definitely, compound (**4**), the 4-nitrooxy derivative, was also investigated through CV (Fig. 6) and as expected, did not present peaks in the less negative part of the CV, showing that the cleavage of C-ONO_2 bond would require higher energy, in the absence of the nitro group. It occurred at *E*pIc = -1.319 V.

Table 1 lists all the electrochemical parameters of the studied compounds (**1-4**).

3.2. Biological studies

3.2.1. Effect of the nitrooxy compound (1) on the plasma concentration of nitrite

The plasma concentration of nitrite was evaluated 5 h after i.p. administration of vehicle (PEG 400 40 %, 4 mL/kg) or the nitrooxy compound (**1**) (150 mg/kg). Plasma nitrite concentration was increased (close to 70 μM) after administration of the nitrooxy compound (**1**) (Fig. 7). This result indicates that the compound possibly releases NO and is in accordance with electrochemical studies, which demonstrated the release of nitrate from the cleavage of the —CH_2-ONO_2 of the nitrooxy compound (**1**). *in vivo*, nitrate can be metabolized to nitrite, followed by reduction to NO [17].

3.2.2. Effects of the nitrooxy compound (1) in experimental models of pain and inflammation

Exposure of mice to the hot plate induced a nociceptive response characterized by jumping off the plate or licking the paws. Previous (30 min) i.p. administration of the nitrooxy compound (**1**) (100 or 150 mg/kg, i.p.), as well as *p.o.* administration of dipyrone (500 mg/kg), used as a positive control, increased the latency of the nociceptive response (Fig. 8). After demonstrating the activity of the nitrooxy compound (**1**) in a model of nociceptive pain, we investigated its effect in a model of inflammatory pain. I.pl. injection of carrageenan (400 μg, 20 μL) induced a marked and long lasting tactile hypersensitivity. Previous (30 min) i.p. administration of the highest dose of the nitrooxy compound (**1**) (150 mg/kg) or dexamethasone (2 mg/kg), used as a positive control, inhibited the tactile hypersensitivity at 1, 3, 5 and 7 h after carrageenan injection. The intermediate dose of the nitrooxy compound (**1**) (100 mg/kg, i.p.) also exhibited activity at 3 and 5 h after injection of the inflammatory stimulus (Fig. 9). I.pl. injection of carrageenan (400 μg, 20 μL), in addition to inducing tactile hypersensitivity, also induced a marked and long lasting paw edema. The paw edema was inhibited by previous (30 min) i.p. administration of the nitrooxy compound (**1**) (50, 100 or 150 mg/kg) or dexamethasone (2 mg/kg), used as a positive control (Fig. 10).

To rule out muscle relaxation or impairment of motor coordination as possible confounding effects when evaluating the activity of the nitrooxy compound (**1**) in the experimental models of pain, we investigated its effect on the performance of the animals in the rotating rod. The time mice spent on the rotating rod was not altered 0.5, 1, 3, 5 and 7 h after administration of the nitrooxy compound (**1**) (150 mg/kg, i.p.), but was markedly reduced by phenobarbital (50 mg/kg, *p.o.*), used as a positive control (Fig. 11). As the nitrooxy compound (**1**) did not affect the time spent by the animals on the rotating rod, it is highly unlikely that the inhibition of the nociceptive behavior in the pain models is due to muscle relaxation or impairment of motor coordination.

The activity of the nitrooxy compound (**1**) in a model of nociceptive pain indicates that it may inhibit nociceptive processing in the central nervous system and/or activation of thermal sensitive nociceptors. In models of nociceptive pain, such as hot plate and tail flick, the immediate nociceptive response displayed by the animals is due to the direct activation of thermal sensitive nociceptors and central processing, without involvement of inflammatory cells or mediators [41,42]. Other studies have demonstrated that known NO donors such as nitroprusside and morpholino-sydnonimine also exhibit activity in models of nociceptive pain [43,44]. On the other hand, the activity of the nitrooxy compound (**1**) in models of inflammatory pain and edema induced by

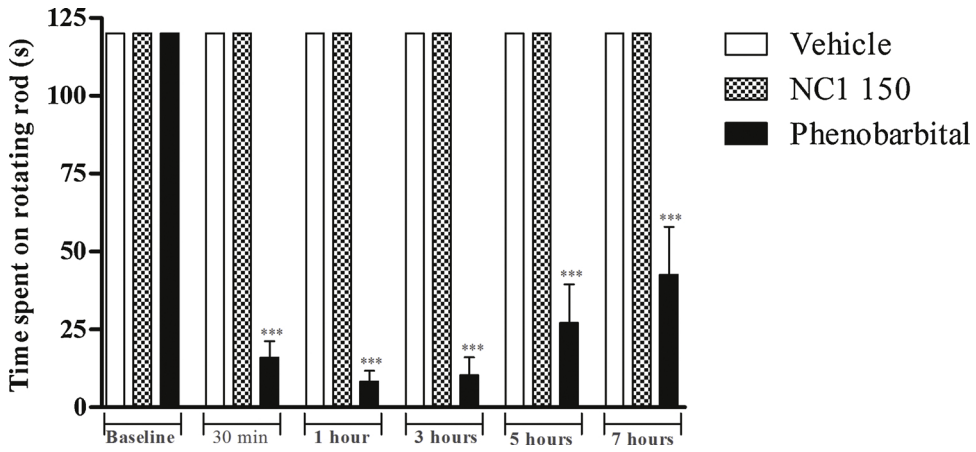


Fig. 11. Effect of the previous (30 min) administration of the nitrooxy compound (1) (NC1; 150 mg/kg, i.p.), phenobarbital (50 mg/kg, *p.o.*) or vehicle (PEG 400 40 %, 4 mL/kg, i.p.) on the time spent by mice on the rotating rod. *** significantly different from vehicle ($p < 0.001$). One-way ANOVA followed by Newman-Keuls test. n = 6.

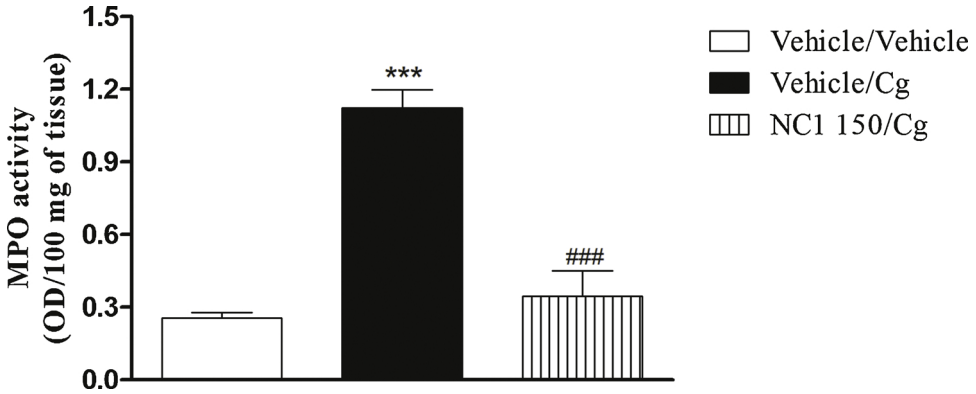


Fig. 12. Effect of the previous (30 min) i.p. administration of the nitrooxy compound (**1**) (NC1; 150 mg/kg) or vehicle (PEG 400 40 %, 4 mL/kg) on the MPO activity induced by i.pl. injection of carrageenan (Cg; 400 μg). MPO activity in the paw tissue was evaluated 5 h after carrageenan injection. *** significantly different from PEG 400/saline ($p < 0.001$). ### significantly different from PEG 400/carrageenan ($p < 0.001$, respectively). One-way ANOVA followed by Newman-Keuls test. n = 6.

carrageenan indicates that this compound may also inhibit the production and/or action of inflammatory mediators. It has also been demonstrated that other NO donors such as nitroprusside, S-nitroso-N-acetyl-DL-penicillamine [45] and nicorandil [46] inhibit the inflammatory edema induced by carrageenan and other stimuli and also the sensitization in a model of inflammatory pain.

Aiming to investigate which mechanisms contribute to the activity of the nitrooxy compound (**1**) in the models of inflammatory pain and edema, we investigated its effects on the production of inflammatory mediators and cell recruitment. Five hours after i.pl. injection of carrageenan (400 μg, 20 μL), MPO activity in the paw tissue was observed (Fig. 12), indirectly indicating an increase in the neutrophil recruitment to the paw tissue after injection of carrageenan. At the same time, IL-6, IL-1β, TNF-α and CXCL-1 concentrations in the paw tissue were increased (Fig. 13). The nitrooxy compound (**1**) (150 mg/kg, -30 min, i.p.) markedly reduced MPO activity (Fig. 12) and IL-6, IL-1β, TNF-α and CXCL-1 concentrations (Fig. 13) in the paw tissue. In addition, treatment with the nitrooxy compound (**1**) also increased the concentrations of IL-10 (Fig. 13).

These results indicate that the activities of the nitrooxy compound (**1**) in the models of inflammatory pain and edema induced by carrageenan may be associated with inhibitory effects on neutrophil recruitment and production of inflammatory mediators. The nitrooxy compound (**1**) markedly reduced MPO activity in the inflamed paw, an indirect evidence of an inhibitory effect on neutrophil recruitment. Wanikiat et al. [47] demonstrated that high concentrations of NO, as observed after treatment with NO releasing compounds, inhibit neutrophil recruitment in vitro. In addition, NO negatively regulates neutrophil secretion [48] and expression of adhesion molecules in these cells [49]. In vivo inhibition of neutrophil recruitment has also been demonstrated after administration of known NO donors such as nicorandil [50] and nitroprusside [51]. These same NO donors also inhibit production of inflammatory cytokines in an experimental model of pleurisy induced by carrageenan [50] and in patients under reperfusion after coronary artery bypass grafting [52]. Finally, the activities of the nitrooxy compound (**1**) in the models of inflammatory pain and edema may also be due to increased production of IL-10, a cytokine with anti-inflammatory activity. IL-10 can act by decreasing the production of proinflammatory cytokines, contributing to the resolution of the inflammatory process [53]. NO can react with soluble guanylyl cyclase producing higher production of cGMP and increasing the production of IL-10, which may contribute to the control of inflammatory processes [54–56].

4. Conclusions

Electrochemical results, obtained in aprotic medium, revealed unequivocally that electron transfer at the nitro group of the doubly redox-functionalized nitrooxy compound (**1**) results in the cleavage of the organic nitrate, potentially resulting in the generation of NO. The nitrooxy compound (**1**) exhibits activities in models of inflammatory pain and edema that may be due to reduced recruitment of neutrophils and production of inflammatory cytokines and increased production of IL-10. These results reinforce the interest in the investigation of NO donor compounds as candidates for analgesic and anti-inflammatory drugs.

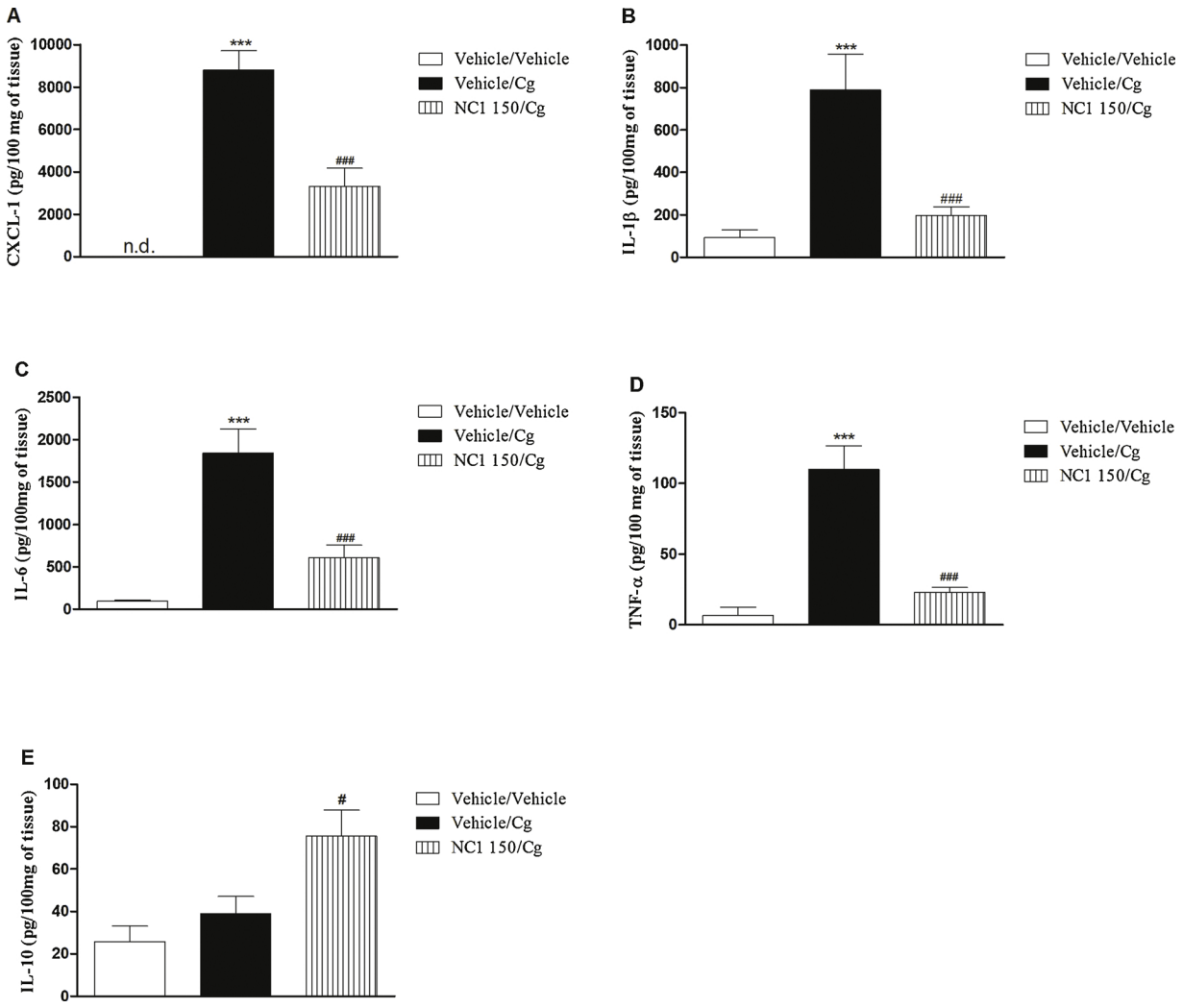


Fig. 13. Effect of the previous (30 min) i.p. administration of the nitrooxy compound (**1**) (NC1; 150 mg/kg) or vehicle (PEG 400 40 %, 4 mL/kg) on the production of CXCL-1 (A), IL-1β (B), IL-6 (C), TNF-α (D) and IL-10 (E) induced by i.pl. injection of carrageenan (Cg; 400 μg). Cytokine concentrations in the paw tissue were evaluated 5 h after carrageenan injection. n.d.: not detectable. *** significantly different from PEG 400/saline ($p < 0.001$, respectively). $^{\#}$ and $^{\#\#\#}$ significantly different from PEG 400/carrageenan ($p < 0.05$ and $p < 0.001$, respectively). One-way ANOVA followed by Newman-Keuls test. $n = 6$.

Declaration of Competing Interest

The authors report no declarations of interest.

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