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Vinpocetine reduces lipopolysaccharide-induced inflammatory pain and neutrophil recruitment in mice by targeting oxidative stress, cytokines and NF-κB

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ABSTRACT

In response to lipopolysaccharide (LPS), tissue resident macrophages and recruited neutrophils produce inflammatory mediators through activation of Toll-like receptor 4 (TLR4)/nuclear factor *kappa* B (NF-κB) signaling pathway. These mediators include inflammatory cytokines and reactive oxygen species that, in turn, sensitize nociceptors and lead to inflammatory pain. Vinpocetine is a nootropic drug widely used to treat cognitive and neurovascular disorders, and more recently its anti-inflammatory properties through inhibition of NF-κB activation have been described. In the present study, we used the intraplantar and intraperitoneal LPS stimulus in mice to investigate the effects of vinpocetine pre-treatment (3, 10, or 30 mg/kg by gavage) in hyperalgesia, leukocyte recruitment, oxidative stress, and pro-inflammatory cytokine production (TNF-α, IL-1β, and IL-33). LPS-induced NF-κB activation and cytokine production were investigated using RAW 264.7 macrophage cell *in vitro*. Vinpocetine (30 mg/kg) significantly reduces hyperalgesia to mechanical and thermal stimuli, and myeloperoxidase (MPO) activity (a neutrophil marker) in the plantar paw skin, and also inhibits neutrophil and mononuclear cell recruitment, superoxide anion and nitric oxide production, oxidative stress, and cytokine production (TNF-α, IL-1β and IL-33) in the peritoneal cavity. At least in part, these effects seem to be mediated by direct effects of vinpocetine on macrophages, since it inhibited the production of the same cytokines (TNF-α, IL-1β and IL-33) and the NF-κB activation in LPS-stimulated RAW 264.7 macrophages. Our results suggest that vinpocetine represents an important therapeutic approach to treat inflammation and pain induced by a gram-negative bacterial component by targeting NF-κB activation and NF-κB-related cytokine production in macrophages.

1. Introduction

Infection by gram-negative bacteria provokes pain and reduces life quality of patients [1–3]. Lipopolysaccharide (LPS) from gram-negative bacteria, or endotoxin, is a prototypical ligand of membrane-bound pattern recognition receptor (PRR) Toll-like receptor 4 (TLR4) and the major constituent of gram-negative bacterial cell wall, covering up to 75% of its outer surface [4]. Neutrophil-derived molecules, and even some antibiotics such as ceftazidime, induce bacterial lysis and, consequently, the release of large amounts of LPS [5]. Thus, despite killing bacteria, bacterial lysis increases the inflammatory pain and leads to an additional post infectious sequel [5]. In this sense, targeting LPS effects is of great clinical importance.

The activation of TLR4 expressed by resident macrophages leads to intracellular signaling pathways that culminate in the activation of nuclear factor *kappa* B (NF-κB) and pro-inflammatory cytokines production, including TNF-α, IL-1β and IL-33 [6,7]. These mediators induce the recruitment of neutrophils that, in turn, produce reactive oxygen species (ROS), such as superoxide anion and its derivatives [8]. Both macrophage- and neutrophil-released cytokines and ROS activate and sensitize (hyperalgesia) nociceptive neurons [9–15]. In this sense, compounds that present both anti-inflammatory and analgesic activities are candidates to treat LPS-induced inflammation and pain, and the TLR4 activation by LPS injection is a useful

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murine model that allows screening and characterization of such compounds.

Ethyl apovincamine-22-oate is also known as vinpocetine, a safe nootropic drug [16] that is widely used to treat cognitive and cerebrovascular disorders, including cerebral infarction and residual cerebral hemorrhage [17,18]. The treatment with vinpocetine showed to be safe and non-toxic even in long-term use [19–21], and no significant side effects related to the treatment have been reported [18,20,22]. The therapeutic effects of vinpocetine are related to its high capacity to protect neuronal cells from the cytotoxic effects of inflammation, oxidative stress [23–26], and ion flux [27,28]. In other tissues, vinpocetine decreased the acute hepatic injury caused by CCl_4 in rats [29], and was gastroprotective in rats [30,31] in addition to the anti-inflammatory activity also reported elsewhere [32,33].

Although vinpocetine exhibits selective inhibitory activity over phosphodiesterase-1 (PDE-1) enzyme [34], its anti-inflammatory effects seem to be PDE-1-independent. Vinpocetine inhibits LPS-induced lung inflammation in mice by targeting NF-κB activation and, consequently, the production of NF-κB-related cytokines TNF-α and IL-1β, as well as the recruitment of polymorphonuclear cells [32]. Corroborating this mechanism of action, vinpocetine exhibited anti-atherogenic effect through inhibition of NF-κB activation and the resulting production of inflammatory cytokines (TNF-α and IL-6), reactive oxygen species, and other inflammatory mediators in a PDE-1-independent manner [33]. In a model of endotoxemia, intraperitoneal administration of vinpocetine reduced hippocampal expression of IL-1β and TNF-α after intraperitoneal stimulus with LPS [35]. In fact, the analgesic activity of intraperitoneal treatment with vinpocetine was described in acetic acid-induced visceral nociception, corroborating the possible use of this compound in inflammatory pain conditions [36]. However, it was not determined if similar analgesic and anti-inflammatory effects could be obtained by oral treatment with vinpocetine in LPS-induced pain. In this study, we used *in vivo* and *in vitro* approaches to investigate the effects of vinpocetine in LPS-induced inflammatory pain and leukocyte recruitment, and their relation to oxidative stress, cytokine production, and NF-κB activation.

2. Materials and methods

2.1. Drugs

Materials were obtained from the sources as follows: commercial tablets of vinpocetine (Vicog®, Marjan Indústria & Comércio Ltda, São Paulo, Brazil), vinpocetine powder at >98% purity (Santa Cruz Biotechnology, Dallas, Texas, USA), LPS from *Escherichia coli* 0111:B4 (Santa Cruz Biotechnology).

2.2. Animals

Male Swiss mice (25–30 g) from the Universidade Estadual de Londrina, Paraná, Brazil, were used in this study. Mice were housed in standard clear plastic cages with free access to food and water and a light/dark cycle of 12–12 h at 21 °C. All behavioral testing was performed between 9 a.m. and 5 p.m. in a temperature-controlled (21 °C) room. Animal care and handling procedures were approved by the Ethics Committee of the Universidade Estadual de Londrina (process number 13278.2011.3). All efforts were made to minimize animals' suffering and to reduce the number of animals used.

2.3. General experimental procedures

Mice received per oral (p.o.) treatment with vinpocetine (3, 10, or 30 mg/kg, 100 μL) or vehicle (sterile saline, 100 μL) 1 h before stimulus injection. Mechanical and thermal hyperalgesia were evaluated 1–5 h after intraplantar (i.pl.) injection of LPS (200 ng in 25 μL of sterile saline). Neutrophil recruitment to the plantar paw skin was evaluated 5 h after LPS i.pl. injection (200 ng in 25 μL of sterile saline) by myeloperoxidase (MPO) activity assay. For oxidative stress tests, samples of plantar paw skin were collected 3 h after LPS i.pl. injection (peak of hyperalgesia). In the peritoneal cavity, leukocyte recruitment and cytokine production were evaluated 2 and 6 h after i.p. injection with LPS (200 ng in 200 μL of sterile saline).

Supernatants and cell lysates from RAW 264.7 cell cultures were collected 6 h after LPS stimulus (1 μg/mL) and used to evaluate cytokine levels (ELISA) and NF-κB activation by luciferase gene reporter assay, respectively.

2.4. Mechanical hyperalgesia test

Mechanical hyperalgesia was measured by an electronic version of von Frey filaments [37]. The test consisted of evoking a hind paw reflex with a hand-held force transducer (electronic anesthesiometer; Insight Equipamentos, Ribeirao Preto, SP, Brazil). After paw withdrawal, the pressure intensity was recorded automatically, and the values were an average of three measurements. Mice were tested before (basal) and after stimulus injection. The results are expressed as delta (Δ) withdrawal threshold (in g), calculated by subtracting the basal mean measurements from the mean measurements obtained at 1, 3, or 5 h after i.pl. stimulus with LPS.

2.5. Hot plate test

Mice were placed on a hot plate apparatus (Hot Plate HP-2002, Insight Equipamentos, Ribeirao Preto, SP, Brazil) maintained at 55 °C. The reaction time was scored when the animal jumped or licked its paws. A maximum latency (cut-off) was set at 30 s to avoid tissue damage [38,39].

2.6. Myeloperoxidase (MPO) activity

The neutrophil recruitment to the plantar paw skin was evaluated by the MPO kinetic-colorimetric assay [40,41]. Samples were homogenized using a tissue-tearor (Biospec®) in ice-cold K_2HPO_4 buffer (400 μL, 50 mM, pH 6.0) containing HTAB (0.5% weight/volume), and the homogenates were centrifuged (16,100*g* × 2 min × 4 °C). The supernatants (30 μL) were mixed with K_2HPO_4 buffer (200 μL, 50 mM, pH 6.0) containing *o*-dianisidine dihydrochloride (0.0167%, w/v) and hydrogen peroxide (0.015%, v/v). The absorbance was determined after 5 min at 450 nm (Multiskan GO Microplate Spectrophotometer, Thermo Scientific, Vantaa, Finland). The results of MPO activity are expressed as the number of neutrophils per mg of tissue by using a standard curve of neutrophils (196–400,000 cells). Neutrophils were harvested from peritoneal cavity 6 h after i.p. stimulus with thiglycolate broth (1 mL, 5%, Becton Dickinson, MD, USA). The number of neutrophils was determined by total counts in Neubauer chamber and differential counts in slices stained by Rosenfelt method. We obtained 94% of neutrophils in a pool of 10 mice. Neutrophils were suspended in K_2HPO_4 buffer containing HTAB and stored at −80 °C until use.

2.7. Oxidative stress tests

2.7.1. ABTS and FRAP assays

The ability of samples to resist oxidative damage was determined by its free radical scavenging (ABTS [2,2′-Azinobis-3-ethyl benzothiazoline 6-sulfonicacid] assay) and ferric reducing (FRAP assay) properties. The tests were adapted to a 96-well microplate

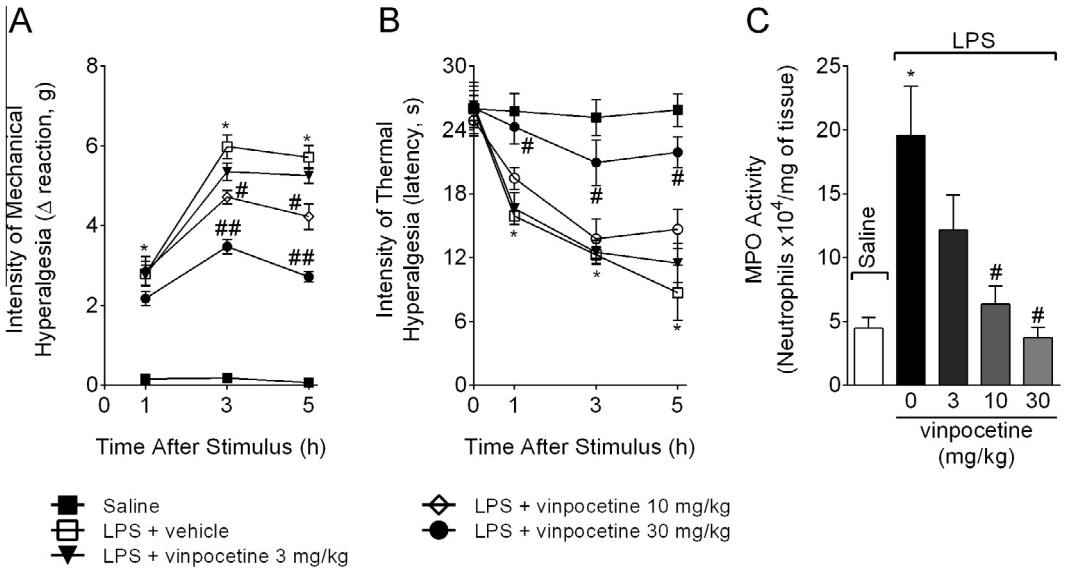

Fig. 1. Vinpocetine inhibited lipopolysaccharide (LPS)-induced hyperalgesia and myeloperoxidase activity (MPO). Mice were treated with vinpocetine (3, 10, or 30 mg/kg, p.o.) or vehicle (saline) 1 h before LPS (200 ng/25 μL) i.pl. injection. Mechanical (Panel A) and thermal hyperalgesia (Panel B) were assessed by electronic von Frey and hot plate apparatus, respectively. Myeloperoxidase (MPO) activity (Panel C) was determined in plantar paw skin samples collected 5 h after LPS injection. Results are presented as means ± SEM of 6 mice per group per experiment, and are representative of 2 separated experiments. $^{*}P < 0.05$ compared with saline group; $^{\#}P < 0.05$ compared with LPS + vehicle group; and $^{\#\#}P < 0.05$ compared with LPS + 10 mg/kg of vinpocetine. One-way ANOVA followed by Tukey's *t* test.

format as previously described [42]. Plantar paw skin samples were collected 3 h after i.pl. stimulus with LPS and homogenized immediately in ice-cold KCl buffer (500 μL, 1.15% w/v). The absorbance of ABTS and FRAP assays were measured at 730 and 595 nm (Multiskan GO Thermo Scientific), respectively, and the results were equated against a standard Trolox curve (0.02–20 nmol).

2.7.2. GSH levels

Samples of plantar paw skin were collected and maintained at −80 °C for 48 h before the test. The GSH levels in samples were measured as described previously [43], and the absorbance was measured at 412 nm (Multiskan GO, Thermo Scientific). Results were equated against a standard GSH curve (0.02–20 nmol) and expressed as nmol of GSH per mg of tissue.

2.7.3. Superoxide anion production

The measurement of superoxide anion production in plantar paw skin homogenates (10 mg/mL in 1.15% KCl) was performed using the nitroblue tetrazolium (NBT) assay adapted to a microplate as described previously [44]. The NBT reduction was measured at 600 nm (Multiskan GO, Thermo Scientific). The tissue weight was used for data normalization.

2.7.4. Nitrite production

Samples from plantar paw skin were collected 3 h after LPS injection, homogenized in 500 μL of saline, and nitrite concentration was determined by the Griess reaction as an indicator of nitric oxide production [45]. Briefly, 100 μL of the homogenate was incubated with 100 μL of Griess reagent for 5 min at 25 °C, and nitrite concentration was determined by measuring the optical density at 550 nm (Multiskan GO, Thermo Scientific) in reference to a standard curve of $NaNO_2$ solution. Results are expressed as μmol of nitrite per mg of tissue.

2.7.5. Lipid peroxidation

Lipid peroxidation was measured by the Thiobarbituric Acid Reactive Substances (TBARS) assay as described previously [43]. Plantar paw skin samples were collected 3 h after i.pl. stimulus with LPS and homogenized immediately in ice-cold KCl buffer (500 μL, 1.15% w/v). Malondialdehyde (MDA) levels, an intermediate product of lipid peroxidation, was determined in samples by the difference between absorbance at 535 and 572 nm (Multiskan GO, Thermo Scientific). Results are reported as nmol of MDA per mg of tissue.

2.8. Total and differential cell counts

Peritoneal cells were harvested with 1.5 mL of PBS 2 h and 6 h after LPS i.p. injection. Aliquots of 20 μL were mixed with 180 μL of Turk's solution, and the total cell counts were performed in Neubauer chamber. Differential cell counts were performed on microscope slides using the Panoptic staining kit for histological analysis (Laborclin, Pinhais, PR, Brazil). The values were expressed as the total number of cells × 10^4 per cavity. Total and differential cell counts were both performed under a light microscope (400× magnification, Olympus Optical Co., Germany).

2.9. Cytokine measurement

Samples from peritoneal exudates were collected by washing peritoneal cavity with 1.5 mL of PBS containing protease inhibitor (1 mM phenylmethanesulfonyl fluoride, Sigma Aldrich) 2 and 6 h after LPS i.p. injection. TNF-α, IL-1β, and IL-33 levels were determined by an enzyme-linked immunosorbent assay (ELISA) using eBioscience kits. The results were equated against standard curves and are expressed as picograms (pg) of each cytokine per cavity.

2.10. Cell culture, luciferase activity and lactate dehydrogenase (LDH) leakage

The murine macrophage cell line RAW 264.7, stably expressing luciferase on NF-κB responsive promoter (pNF-κB-Luc) [46], was routinely cultured in Dulbecco's modified Eagle's medium supplemented (10% fetal bovine serum and penicillin–streptomycin) at 37 °C in a humidified atmosphere of 5% CO_2. For the luciferase reporter assay, RAW 264.7 macrophages (3 × 10^5 cells/well) were grown in 24 well plates. After culturing for 24 h, cells were treated with different drug concentrations and stimulated with lipopolysaccharide (1 μg/mL). Intracellular contents were

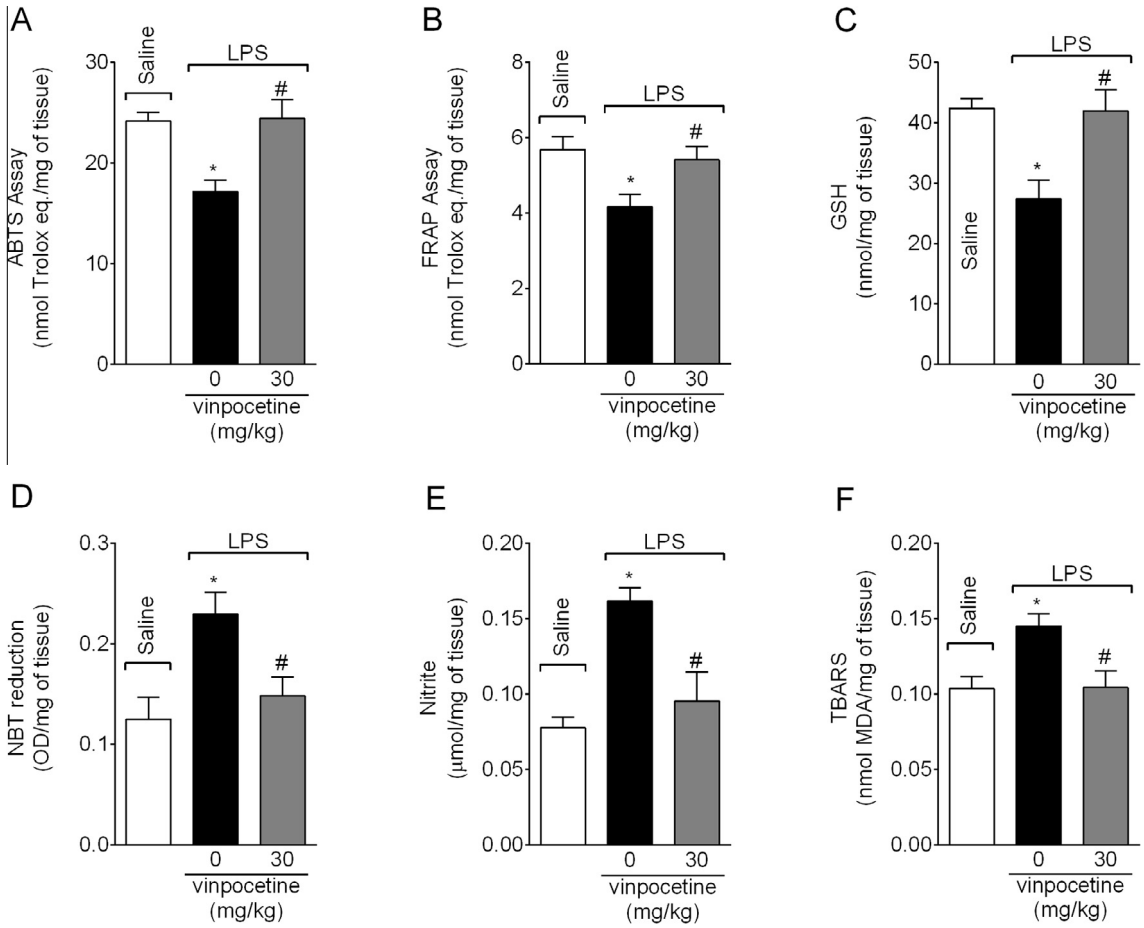

Fig. 2. Vinpocetine reduces LPS-induced oxidative stress in plantar paw skin. Mice were treated with vinpocetine (30 mg/kg, p.o.) or vehicle 1 h before i.pl. stimulus with LPS (200 ng/25 µL). Plantar paw skin samples were collected 3 h after LPS injection and used to measure the ability to scavenge ABTS free radical (Panel A), ferric reducing ability potential (FRAP) (Panel B), reduced gluthathione (GSH) levels (panel C), and superoxide anion (Panel D), nitrite (Panel E), and malondialdehyde (MDA) (Panel F) production. Results are presented as means ± SEM of 6 mice per group per experiment, and are representative of 2 separated experiments. $^{*}P < 0.05$ compared to saline group, and $^{\#}P < 0.05$ compared to LPS + vehicle group. One-way ANOVA followed by Tukey's *t* test.

extracted in lysis buffer (TNT), and the luciferase activity in cell lysates was determined on a luminometer (Victor X5, PerkinElmer, Waltham, MA) using the Dual Luciferase Reporter assay system (Promega, Wisconsin, USA). Data are expressed as a ratio of relative luminescence units (RLU). LDH leakage was measured in supernatants to determine cytotoxicity using the Cytotoxicity Detection Kit^{PLUS} (Roche Applied Science, Mannheim, Germany) according to the manufacture's directions.

2.11. Data analyses

Results are presented as means ± SEM of measurements made on 6 mice or 4 wells per group per experiment and are representative of two independent experiments. Two-way ANOVA was used to compare the groups and doses at all times when the parameters were measured at different times after the stimulus injection. The analyzed factors were treatments, time, and time versus treatment interaction. One-way ANOVA followed by Tukey's *t*-test was performed for each time-point. $P < 0.05$ was considered significant.

3. Results

3.1. Vinpocetine reduces LPS-induced hyperalgesia and myeloperoxidase (MPO) activity

Mice were treated with vinpocetine (3, 10, or 30 mg/kg, p.o.) 1 h before LPS (200 ng/25 µL) i.pl. injection. Mechanical (Fig. 1A) and thermal (Fig. 1B) hyperalgesia were evaluated at the indicated time points after LPS injection. Immediately after the last measurement (5 h), mice were euthanized and plantar paw skin samples were collected for MPO activity measurement (Fig. 1C), an indicator of neutrophil recruitment. LPS injection induced mechanical (Fig. 1A) and thermal (Fig. 1B) hyperalgesia at all evaluated time points, and also increased MPO activity 5 h after stimulus (Fig. 1C). No inhibitory effect was observed in mice treated with vinpocetine at the dose of 3 mg/kg (Fig. 1). On the other hand, treatment with 10 mg/kg of vinpocetine inhibited LPS-induced mechanical hyperalgesia at 3 and 5 h (Fig. 1A), and MPO activity (Fig. 1C), but no effect was observed regarding thermal hyperalgesia (Fig. 1B). The dose of 30 mg/kg of vinpocetine reduced up to 53% of mechanical hyperalgesia at 3 and 5 h (Fig. 1A), and reduced up to 77% of thermal hyperalgesia at all evaluated time points (Fig. 1B). Moreover, vinpocetine at the dose of 30 mg/kg abolished LPS-induced increase of MPO activity (Fig. 1C). Thus, the dose of 30 mg/kg of vinpocetine was selected for the next experiments.

3.2. Vinpocetine reduces oxidative stress induced by LPS in plantar paw skin

Mice were treated with vinpocetine (30 mg/kg, p.o.) 1 h before i.pl. stimulus with LPS (200 ng/25 µL). Plantar paw skin samples were collected 3 h after stimulus injection and used to evaluate the oxidative stress (Fig. 2). LPS injection reduced the levels of endogenous antioxidants as observed in ABTS free radical

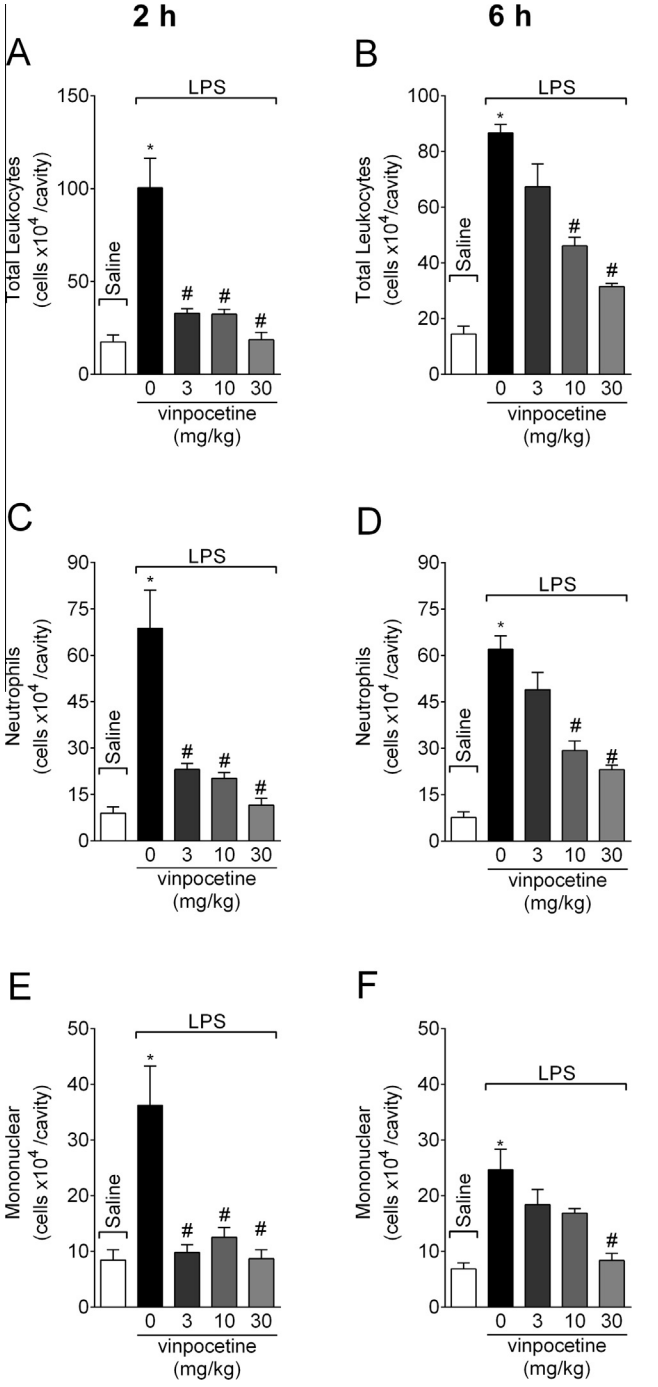

Fig. 3. Vinpocetine inhibited LPS-induced leukocyte migration in the peritoneal cavity. Mice were treated with vinpocetine (3, 10, or 30 mg/kg, p.o.) or vehicle (saline) 1 h before i.p. injection of LPS (200 ng/200 μL). Total leukocytes (Panels A and B), neutrophils (Panels C and D), and mononuclear cells (Panels E and F) were assessed 2 h and 6 h after LPS injection in peritoneal samples. Results are presented as means ± SEM of 6 mice per group per experiment, and are representative of 2 separated experiments. $^{*}P < 0.05$ compared to saline group, and $^{\#}P < 0.05$ compared to LPS + vehicle group. One-way ANOVA followed by Tukey's *t* test.

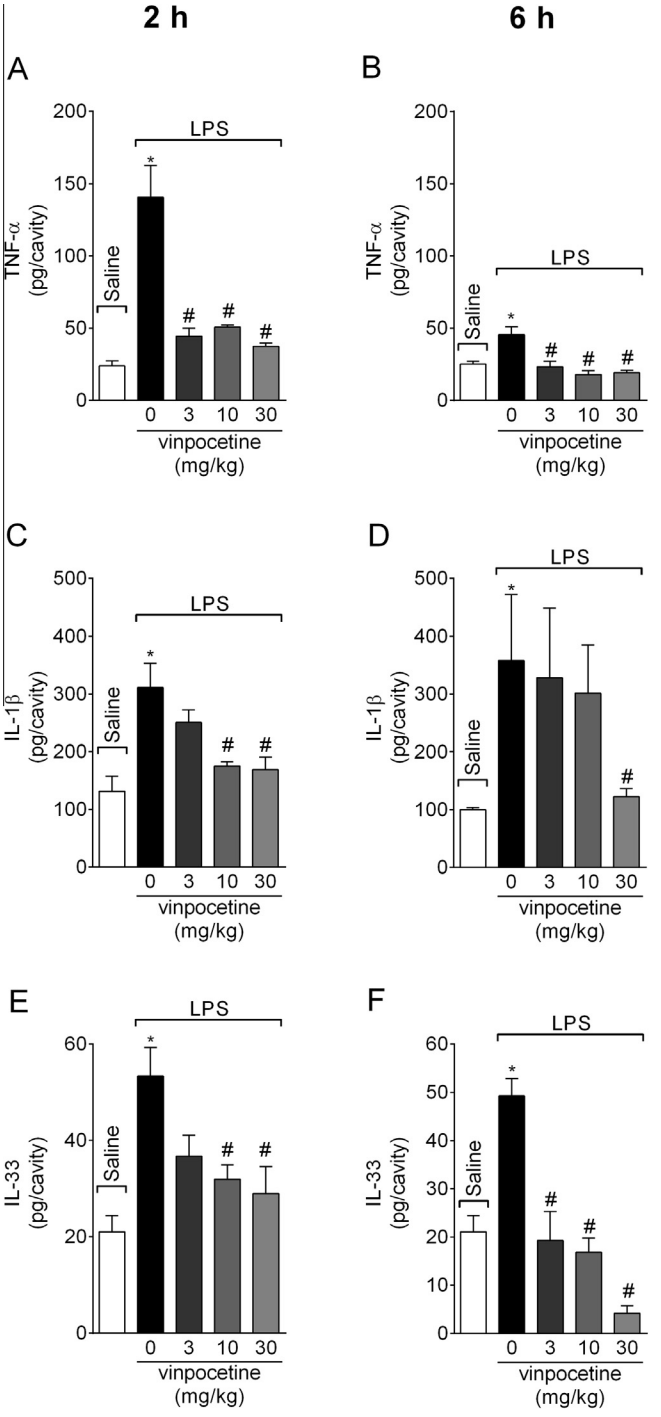

Fig. 4. Vinpocetine inhibited LPS-induced cytokine production in the peritoneal cavity. Mice were treated with vinpocetine (3, 10, or 30 mg/kg, p.o.) or vehicle (saline) 1 h before i.p. injection of LPS (200 ng/200 μL). Peritoneal exudate samples were collected 2 h (Panels A, C and E) and 6 h (Panels B, D and F) after LPS injection, and the levels of TNF-α (Panels A and C), IL-1β (Panels B and D) and IL-33 (Panels E and F) were determined. Results are presented as means ± SEM of 6 mice per group per experiment, and are representative of 2 separated experiments. $^{*}P < 0.05$ compared to saline group, and $^{\#}P < 0.05$ compared to LPS + vehicle group. One-way ANOVA followed by Tukey's *t* test.

scavenging ability (Fig. 2A), FRAP (Fig. 2B), and GSH (Fig. 2C) assays. These results are in line with the increases in superoxide anion (Fig. 2D), nitrite (Fig. 2E), and MDA (Fig. 2F) production that were induced by LPS injection. The treatment with vinpocetine, in turn, inhibited up to 78% and 80% of LPS-induced superoxide anion (Fig. 2D) and nitrite (Fig. 2E) production, respectively. The oxidative stress was also inhibited up to 100% by vinpocetine treatment (Fig 2A–E).

3.3. Vinpocetine reduced LPS-induced leukocyte recruitment in the peritoneal cavity

Mice were treated with vinpocetine (3, 10, or 30 mg/kg, p.o.) 1 h before i.p. injection of LPS (200 ng/200 μL), and the leukocyte

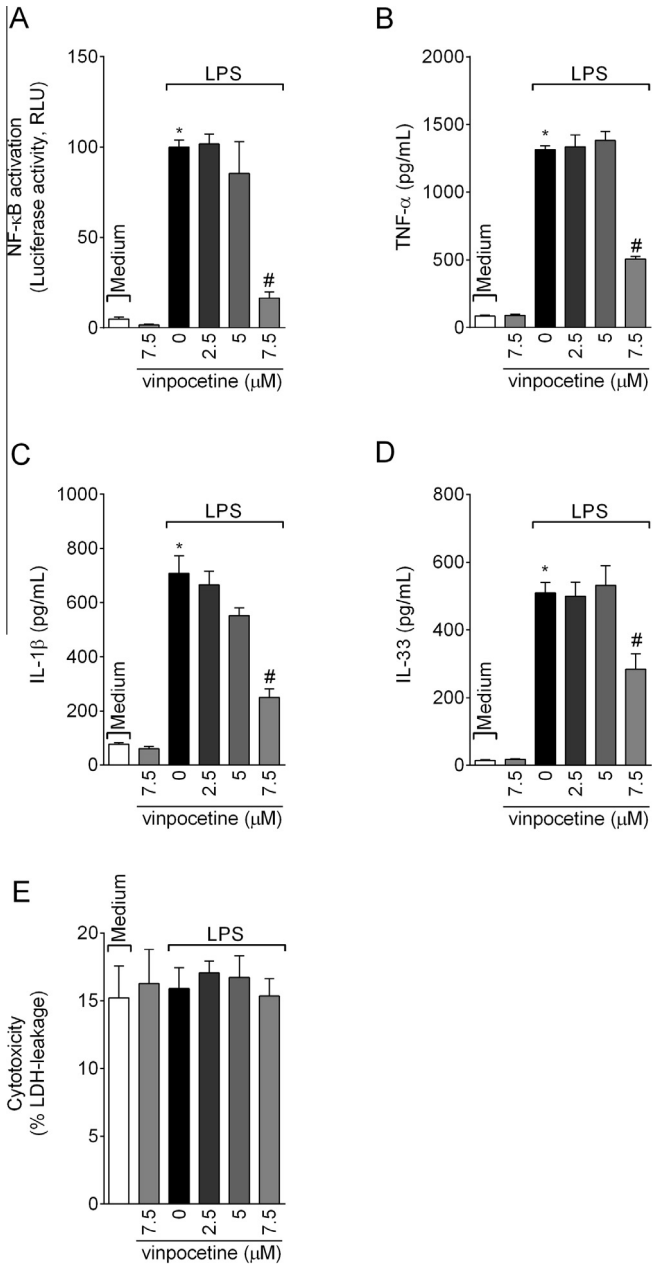

Fig. 5. Vinpocetine inhibited LPS-induced NF-κB activation and IL-1β, TNF-α and IL-33 production by macrophages. RAW 264.7 macrophages were treated with vinpocetine (2.5–7.5 μM) or vehicle (medium) 1 h before stimulus with LPS (1 μg/mL). Luciferase activity in cell lysates (Panel A), cytokine production (Panels B–D) and LDH levels (Panel E) in supernatants were determined 6 h after LPS stimulus. Results are presented as means ± SEM of 4 wells per group per experiment, and are representative of 2 separated experiments. $^{*}P < 0.05$ compared to medium group, and $^{\#}P < 0.05$ compared to LPS + 0 mg/kg group. One-way ANOVA followed by Tukey's *t* test.

recruitment in peritoneal cavity was evaluated 2 h (Fig. 3A, C and E) and 6 h (Fig. 3B, D and F) later. LPS injection induced significant increase of leukocyte recruitment at 2 h (Fig. 3A) and 6 h (Fig. 3B). All doses of vinpocetine were capable of reducing LPS-induced leukocyte recruitment at 2 h (Fig. 3A), but only the doses of 10 and 30 mg/kg of vinpocetine showed efficacy at 6 h (Fig. 3B). At 2 h after LPS injection, all doses of vinpocetine were capable of reducing neutrophil (Fig. 3C) and mononuclear cell (Fig. 3E) recruitment. At 6 h, neutrophil recruitment was reduced by treatment with vinpocetine at doses of 10 and 30 mg/kg (Fig. 3D), whilst mononuclear cell recruitment was reduced only by vinpocetine at the dose of 30 mg/kg (Fig. 3F).

3.4. Vinpocetine inhibits LPS-induced cytokine production in the peritoneal cavity

Mice were treated with vinpocetine (3, 10 or 30 mg/kg, p.o.) 1 h before i.p. injection of LPS (200 ng/200 μL). The levels of TNF-α, IL-1β, and IL-33 were measured 2 and 6 h after LPS injection. LPS injection induced TNF-α, IL-1β, and IL-33 production at 2 h (Fig. 4A, C, and E, respectively) and 6 h (Fig. 4B, D, and F, respectively). The treatment with vinpocetine at the dose of 30 mg/kg inhibited TNF-α, IL-1β, and IL-33 production at 2 h (Fig. 4A, C, and E) by up to 88%, 79% and 76%, respectively, and at 6 h (Fig. 4B, D, and F) by up to 100%, 91% and 100%, respectively. At the dose of 10 mg/kg, vinpocetine also reduced the production of these cytokines, except by IL-1β at 6 h (Fig. 4D), while the treatment with vinpocetine at the dose of 3 mg/kg reduced TNF-α levels at 2 h (Fig. 4A) and 6 h (Fig. 4B), and reduced IL-33 levels at 6 h (Fig. 4F).

3.5. Vinpocetine inhibits LPS-induced NF-κB activation and cytokine production in RAW 264.7 macrophages

RAW 264.7 macrophages (5×10^5 cells/well), stably expressing luciferase on NF-κB responsive promoter, were pre-treated with vinpocetine (2.5–7.5 μM) 1 h before LPS treatment (1 μg/mL). NF-κB activation (luciferase activity in cell lysates) and cytokine release (ELISA in supernatants) were measured 6 h after LPS treatment. The pre-treatment with vinpocetine at the concentration of 7.5 μM reduced LPS-induced NF-κB activation (Fig. 5A) by 87%, and the release of TNF-α (Fig. 5B), IL-1β (Fig. 5C), and IL-33 (Fig. 5D) by 65%, 72%, and 45%, respectively. Neither LPS nor vinpocetine were cytotoxic as determined by LDH assay (Fig. 5E).

4. Discussion

As the most abundant constituent of gram-negative bacterial cell wall, LPS is released in large amounts after lysis of bacteria induced either by endogenous (e.g. complement, lysozyme) and exogenous (bacteriolytic antibiotics) molecules. LPS plays a major role in the pathophysiology of infectious diseases due to its high pro-inflammatory activity [4,6,7,47]. Macrophages are tissue-resident cells from innate immune system capable of recognizing pathogens via pattern recognition receptors (PRR) and are crucial to protect the host against bacterial infections. TLR4 is a PRR responsible for recognizing LPS, and consequent activation of macrophages triggers inflammation through the production and release of pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-33 [7].

The anti-inflammatory activity of vinpocetine was recently demonstrated to be dependent on inhibition of NF-κB pathway in TNF-α-stimulated macrophages [32]. In line with this previous work, our results show that vinpocetine inhibited LPS-induced NF-κB activation and TNF-α, IL-1β, and IL-33 production by macrophages. When these cytokines activate endothelial cells, they up-regulate the expression of adhesion molecules promoting neutrophil recruitment to tissue [48–50]. Importantly, neutrophils are the major sources of myeloperoxidase, a bacteriolytic enzyme that increases LPS release during gram-negative bacteria infection [5]. Herein, we used *in vivo* approaches to show that vinpocetine treatment reduces neutrophil recruitment (into the peritoneal cavity) and the activity of myeloperoxidase (in plantar paw skin) induced by LPS in mice. Taking into account the similar temporal pattern of inhibition between leukocyte recruitment and cytokine production

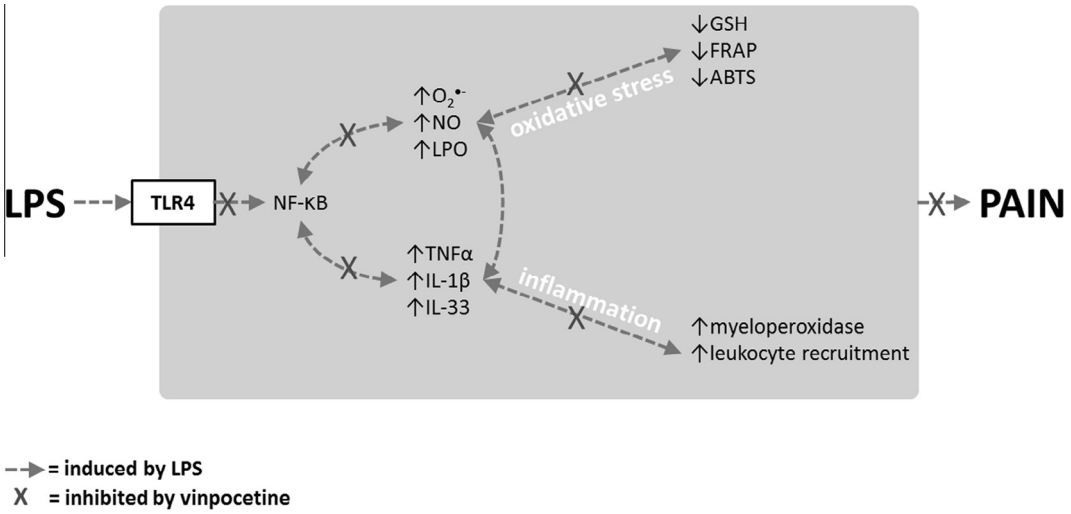

Fig. 6. Proposed targets of vinpocetine in LPS-induced inflammation, oxidative stress and pain. LPS activates TLR4 on resident inflammatory cells, which leads to activation of NF-κB and consequent production of inflammatory cytokines (TNFα, IL-1β, IL-33), superoxide anion ($O_2^{\bullet-}$), nitric oxide (NO) and lipid peroxidation (LPO), and diminished levels of reduced glutathione (GSH), reduced ability to reduce iron (FRAP) and to scavenge ABTS free radicals [6–8]. The inflammatory molecules are released and induce inflammation (leukocyte recruitment and myeloperoxidase activity) and pain [15–24].

(TNF-α, IL-1β and IL-33), and the effects of vinpocetine on macrophages *in vitro*, we suggest that vinpocetine can target tissue resident macrophages activation reducing LPS-induced inflammation and pain. Consistent with our results, vinpocetine inhibits neutrophil recruitment and expression of TNF-α, IL-1β, and MIP-2 in a mouse model of lung inflammation induced by LPS [32].

Neutrophils produce large amounts of superoxide and peroxynitrite anion (formed from the reaction of superoxide anion with nitric oxide) and play a central role in oxidative stress and tissue damage during inflammation. Vinpocetine inhibited oxidative stress as observed by enhanced ability to scavenge ABTS radical, ferric reducing ability, prevention of GSH depletion, reduced superoxide anion and nitric oxide production, and lipid peroxidation. The oxidative stress sustains inflammatory response by mechanisms including NF-κB activation and the production of IL-1β and TNF-α. Additionally, these cytokines contribute to increase tissue damage by extending the survival of neutrophils [51]. Considering the mechanisms described above (Fig. 6), we conclude that vinpocetine exhibits a strong anti-inflammatory effect and represents a safe approach [21,52] to control inflammation-related disorders, including mononuclear/neutrophil recruitment and inflammatory pain.

LPS induces acute hyperalgesia in mice in a MyD88 (myeloid differentiation primary response 88)-dependent and TRIF-independent manner [15], suggesting the role of the NF-κB pathway and pro-inflammatory cytokines in the nociceptive sensitization process. Furthermore, vinpocetine inhibits LPS-induced lung inflammation and TNF-α-induced cellular activation by targeting IKK independently of PDE-1 [32]. TNF-α, IL-1β, and IL-33 are pro-hyperalgesic cytokines due to their capacity of acting on nociceptive neurons to induce its depolarization and/or to induce further production of hyperalgesic molecules capable of activating nociceptive neurons [11,14,53–55]. Superoxide and peroxynitrite also sensitize nociceptive fibers and induce pain [9,56–60]. Thus, it is reasonable to consider that vinpocetine inhibited LPS-induced inflammatory pain by reducing the production of such hyperalgesic mediators. It has been described that even when low doses of LPS (0.4 ng/kg) reaches the blood, it increases systemic immune activation and the pain sensitivity [61], corroborating the idea that small peripheral immune activation is sufficient to modulate pain thresholds [62]. Moreover, bacterial components (e.g. *N*-formylated peptides and LPS) activate nociceptors in a calcium flux-dependent manner [63,64]. Vinpocetine also acts directly on neurons to block sodium currents and the resulting increase of intracellular calcium levels [65–68], suggesting that these activities may represent contributing mechanisms by which this compound inhibits LPS-induced neuronal activation. Additionally, vinpocetine inhibits the PDE-1 activation and thus increases cGMP levels, which is a mechanism of action of analgesics and nitric oxide donors [69–72]. On the other hand, the PDE-1 inhibition by vinpocetine also leads to increases in cAMP levels that, in turn, induce protein kinase A (PKA) and PKCε activity in neurons, a mechanism that could increase pain sensation [73,74]. Taking into account the role of nociceptor sensory neuron activation in neurogenic inflammation [64], the effect of vinpocetine in this context needs to be further investigated.

5. Conclusion

Pain is one of the most prominent complains of patients with infectious diseases, and hyperalgesic cytokines play an important role in this condition. Macrophages and neutrophils are the major sources of these endogenous hyperalgesic mediators. We found that vinpocetine inhibited LPS-induced NF-κB activation and cytokine production (TNF-α, IL-1β, and IL-33) in macrophages *in vitro*, highlighting its anti-inflammatory activity. Similar effects were observed *in vivo*, where this compound also reduced the production of all evaluated cytokines after LPS intraperitoneal injection. The recruitment of neutrophils and monocytes to the peritoneal cavity were also inhibited, which lined up well with the inhibition of superoxide anion and nitric oxide production, as well as the resulting oxidative stress (reduction in antioxidant capacity, depletion of GSH, and induction of MDA) in the plantar paw skin (Fig. 6). The present study did not address the direct acting effects of vinpocetine on nociceptors or its effects on central nervous system, which also represent an important analgesic mechanism of action and were investigated elsewhere [66,75]. Finally, we suggest that vinpocetine, which is widely used to treat cognitive disorders, also represents a promising and safe approach to treat inflammatory pain.

Conflict of Interest

The authors declare no conflict of interest.

Transparency Document

The Transparency document associated with this article can be found in the online version.

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