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Carvacrol suppresses LPS-induced pro-inflammatory activation in RAW 264.7 macrophages through ERK1/2 and NF-kB pathway

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ABSTRACT

Macrophages are immune system cells that respond to various pathogenic insults. The recognition of antigens is performed through receptors such as TLR4 and RAGE, which recognize pathogen-associated patterns (PAMPs), including lipopolysaccharide (LPS) from Gram-negative bacteria. Carvacrol (CAR) is a phenolic compound found in some essential oils commonly used in folk medicine for treatment of inflammation-related diseases. Previous works observed strong antioxidant actions and some anti-inflammatory effects by CAR in *in vivo* and *in vitro* assays. However, the potential pharmacological application of CAR remains limited as details on its mechanisms of action are still missing. Here we investigated the molecular pathways by which CAR acts on LPS-mediated pro-inflammatory activation of RAW 264.7 macrophages. CAR 100 μM protected cells against loss of cell viability induced by LPS (1 μg/mL). Pre-incubation with CAR prevented LPS-induced ERK1/2 phosphorylation, but it had no effect on p38 and JNK activation. The effect of LPS on NF-kB (p65) translocation from cytoplasm to nucleus was inhibited by CAR, as well as NF-kB transcriptional activation. Moreover, LPS-elicited release of TNF-α and IL-1β were inhibited by CAR, as well as activation of phagocytic activity. Such effects may be related to the antioxidant effect of CAR, as the LPS-induced increase in reactive species (RS) production (assessed by DCFH oxidation) and nitric oxide (NO) production (assessed by nitrite quantification) were inhibited by CAR. Altogether, these results demonstrate that CAR exerts relevant anti-inflammatory actions through a cellular mechanism involving ERK1/2 and NF-kB inhibition and possibly related to the antioxidant properties of this phenolic compound.

1. Introduction

Inflammation is a key response involving the participation of a variety of chemical mediators and signaling pathways. During this process, macrophages act as major mediators of the inflammatory response against exogenous pathogens [1]. Macrophages are phagocytic cells derived from blood monocytes and play essential roles in innate and adaptive immunity, acting as antigen presenters and being part of the primary response to a new antigen. These cells are outfitted with pathogen-associated molecular pattern (PAMP) receptors, such as Toll-like receptors (TLRs) and receptor for advanced glycation end-products (RAGE), capable of recognizing extracellular antigens, such as bacterial lipopolysaccharide (LPS) [2]. Upon binding to macrophage PAMP receptors, LPS triggers different intracellular signal pathways that are involved in the inflammatory burst, including cytokines and reactive oxygen/nitrogen species (RS), such as superoxide radicals and nitric oxide (NO) [3,4].

Following receptor binding, LPS also triggers rapid cell signaling responses through activation of protein kinase-dependent pathways; these, in turn, result in the activation of enzymes and transcription

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factors involved in the immune response. Effects of LPS include the activation of ERK1/2, JNK and p38 MAP kinases (MAPKs), which ultimately control the activity of transcription factors that modulate the expression of inducible NO synthase (iNOS), cyclooxygenase-2 (COX-2), TNF-α, IL-1α, IL-1β and IL-6, among other inflammation modulators [5–10]. The NF-kB transcription factor is one of the main modulators of pro-inflammatory gene transcription activated by LPS through TLR4 and RAGE [2,11].

A variety of medicinal and herbal plants present therapeutic activities due to the biological actions of essential oils. Carvacrol (5-isopropyl-2-methylphenol; CAR) is a phenolic monoterpene commonly found in the essential oils of the Laminaceae family, including plants from the *Origanum* and *Thymus* genders [12,13]. Species from these genders are enriched in CAR and are largely used in traditional medicine to treat the flu, cold, bronchitis and coughs [14]. Previous studies demonstrated antinociceptive and anti-inflammatory activities of CAR, which were suggested to be mediated by an inhibitory effect on TNF-α and NO [15,16]. This anti-inflammatory effect of CAR has been ascribed to a capacity to inhibit the synthesis of prostaglandins, suppressing COX-2, inducing the activity of PPAR-α and γ [17,18], stimulating IL-10 action and reducing the local levels of IL-1β and PGE2 [19]. Besides, the high antioxidant capacity of this compound may also be related to this anti-inflammatory activity [15]. Although these studies demonstrated that CAR presents anti-inflammatory properties, the cellular/molecular mechanisms of its action are not well understood.

Previously, it was demonstrated that CAR reduces inflammatory responses through modulation of TNF-α and IL-1β, c-Fos, NFAT-1 and NFAT-2 mRNA expression, inhibited JNK and STAT3 and stimulated p38 phosphorylation in J774.1 mouse macrophage cells stimulated with LPS [20]. In the present work, we evaluated the protective effect of CAR against LPS-induced pro-inflammatory activation in RAW 264.7 macrophages. We observed that CAR exerted anti-inflammatory effects at multiple cellular targets and these effects are probably associated to CAR antioxidant properties.

2. Material and methods

2.1. Chemicals

Murine macrophage cell line RAW 264.7 was obtained from Rio de Janeiro Cell Bank (BCRJ — Rio de Janeiro, Brazil). Dulbecco's modified Eagle's medium (DMEM), antibiotic antimycotic solution, lipopolysaccharide (LPS), Carvacrol (CAR) and TNF-α (RAB0479-1KT), IL-1β (RAB0272-1KT) and anti-β-tubulin III rabbit polyclonal (T 2200) were acquired from Sigma-Aldrich® Chemical Co. (St. Louis, MO, USA). Electrophoresis and western blot materials were purchased from BioRad (Hercules, USA), GE Healthcare Brazilian Headquarter (São Paulo, Brazil). Anti-Lamin B goat polyclonal (SC 6217) was from Santa Cruz Biotechnology, Inc. (Texas, USA). Anti-β-actin rabbit polyclonal (#4967), anti-p-ERK1/2 (#9101S), anti-ERK1/2 (#9102S), anti-p-p38 (#9211), anti-p38 (#9212) anti-p-SAPK/JNK (Thr183/Tyr185) (#9255), anti-SAPK/JNK (#9252), NFκB-p65 (#8242), were obtained were from Cell Signaling Technology® (Beverly, USA). HRP conjugated goat anti-rabbit and goat-mouse secondary antibodies. For each assay, CAR (1 M) was dissolved in DMSO (100%) and serial dilutions were obtained from this stock solution. Therefore, at the highest concentration of UA in these assays 100 μM, concentration of the vehicle DMSO would correspond to 0.1%.

2.2. Cell culture and treatments

Macrophages RAW 264.7 cells were grown in 75-cm^2 flasks maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and 1× antibiotic antimycotic solution (Sigma-Aldrich®) at 37 °C in 5% CO_2 humidified air. The cells were plated respectively at a density of 1×10^5 cells/mL in 96- and 6-well plates for 24 h. For treatments, CAR and LPS were dissolved in the culture medium. After treatments, supernatants were used for lactate dehydrogenase (LDH) activity, measurement of nitrite content and levels of cytokines, and the cells were used for different assays. For viability assay, morphology, production of RS and detection of RAGE, cells were pretreated with CAR at concentration 100 μM in DMSO for 1 h and then stimulated with LPS (1 μg/mL) for additional 24 h. For rapid cellular responses, such as MAPK activation and nuclear translocation of NF-κB, DMEM 10% FBS was first replaced for fresh DMEM 1% FBS. After 3 h, cells were pretreated with CAR 100 μM for 1 h and then LPS (1 μg/mL) was added to the culture medium for 15 min to assess MAPK phosphorylation, 1 h to assess NF-kB nuclear translocation and 12 h to perform the NF-kB gene reporter assay.

2.3. Cell viability and morphology

First, different concentrations of CAR between 75 μM and 300 μM were tested for 24 h in order to assess toxicity through determination of the half-maximum Growth Inhibitory concentration (GI_{50}) of CAR by MTT assay and the half-maximum Effective Dose (ED_{50}) of CAR in detection of LDH activity in the extracellular medium. The percentage of viable cells after treating with either CAR alone or in the presence of LPS (1 μg/mL) during 24 h, was quantified by the MTT reduction assay as previously described. This technique is based on the ability of viable cells to reduce MTT to a formazan precipitate [21]. Optical density was measured at 550 nm and 630 nm using Spectra MAX i3 (Molecular Devices). Immediately after treatments, the culture medium was removed and used for assay of LDH activity using a commercial kit (Labtest®, MG, Brazil). This assay is used to measure LDH activity in the extracellular medium, which is an indicator of cell membrane rupture. Alterations in the morphology of the cells was assessed by phase-contrast microscopy (Mycroscopy EVOS® FL Auto Imaging System — AMAFD1000 Thermo Fisher Scientific; MA, USA).

2.4. Cellular fractionation

To evaluate an essential step of NF-kB, nuclear translocation of p65 was assessed as previously described [22]. After treatments, the cytosolic and nuclear fractions were isolated. Briefly, culture medium was removed and cells washed with ice-cold PBS, then scraped in PBS and centrifuged (500 ×*g* for 5 min). Next, cells were suspended in hypotonic lysis buffer containing protease and phosphatase inhibitors and incubated on ice for 15 min. The homogenate was centrifuged (13,000 ×*g*, 30 s), therefore separating the nuclear extracts (pellet) from the cytoplasmic extracts (supernatant). The isolate nuclear protein extracts were resuspended in ice-cold hypertonic lysis buffer and incubated for 40 min, followed by centrifugation at (13,000 ×*g*, 10 min, 4 °C), and supernatants containing nuclear proteins were collected. The protein content was measured by the Bradford method [23]. Nuclear and cytosolic NF-kB immunocontent were analyzed by western blot in relation to β-actin and Lamin B, constitutive proteins of respective cellular fractions used as controls.

2.5. NF-kB reporter assay

RAW 264.7 cells were seeded in 96-well plates at 1×10^4 cells per well. After 24 h, they were transfected with 100 ng of a vector containing a responsive element to NF-kB driving firefly luciferase (pGL4.32 Luc2P-NF-κB®; Promega) and 10 ng constitutive *Renilla*-luciferase construct (pRL-TK®, Promega) per well using ViaFect® reagent (Promega) and Opti-MEM as previously described [24]. Thereafter, macrophages were pre-treated with CAR for 1 h and then treated overnight with LPS. The cells were lysed and luciferase activity was assessed using the Dual-Glo® Luciferase Assay System (Promega). Results were expressed as the ratio between firefly and *Renilla* luciferases and expressed as relative to control groups (considered as RLU = 100).

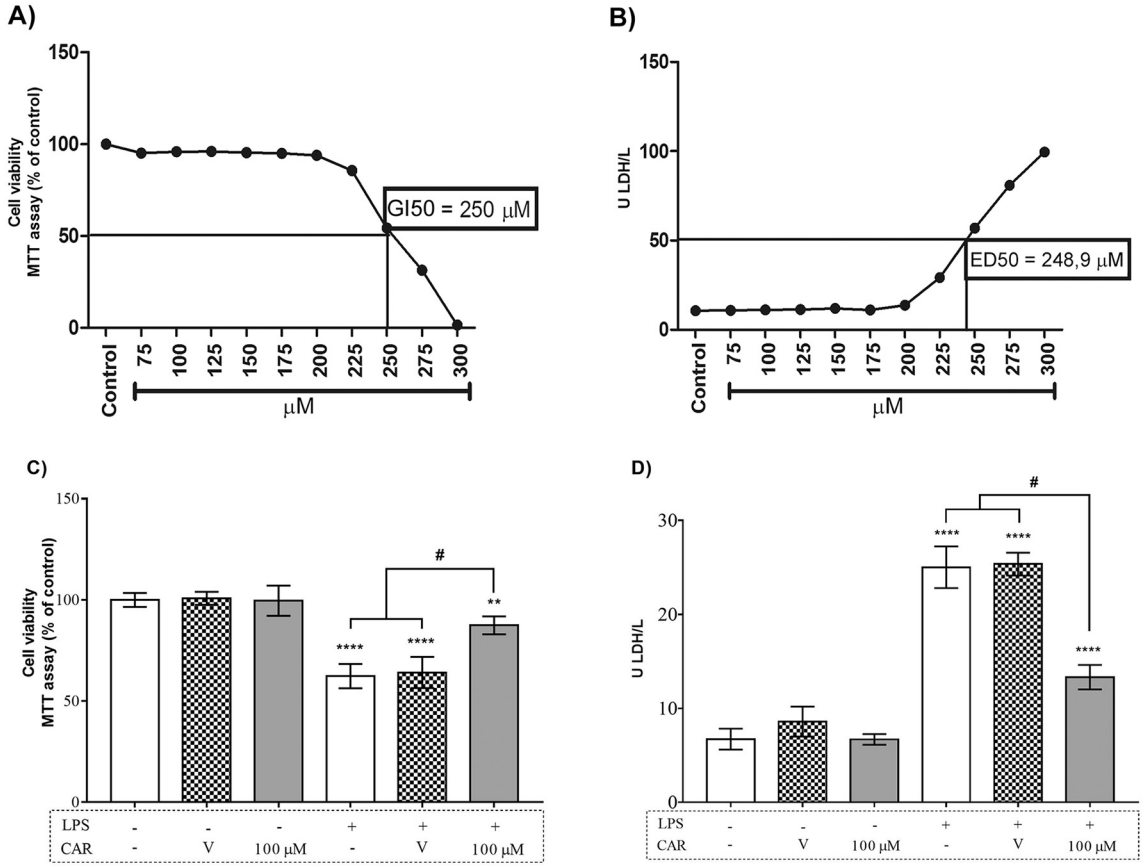

Fig. 1. Cell viability measurements in the presence of CAR. RAW 264.7 cells were treated for 24 h with increasing concentrations of CAR (75–300 μM) and cell viability was assessed by MTT reduction (A) and LDH release (B) assays. The effect of CAR 100 μM on MTT reduction (C) and LDH release (D) of cells treated with LPS 1 μg/mL was tested. CAR, carvacrol; V, vehicle (DMSO). Statistical analysis ($n = 3$ in biological and experimental triplicate, one-way ANOVA followed by Tukey's) was performed: $^{**}p < 0.01$, $^{****}p < 0.0001$ relative to control; $^{\#}p < 0.0001$ relative to LPS treated groups.

2.6. Enzyme-linked immunosorbent assay (ELISA) determination of cytokines

The levels of cytokines in the incubation medium were quantified using kits to measure TNF-α (RAB0479-1KT) and IL-1β (RAB0272-1KT) from Sigma-Aldrich® (MO, USA). After treatments, the culture medium was collected and incubated in ELISA plates, then further processed according to the manufacturer's protocol.

2.7. Western blot

The immunoblotting was performed as previously described [25]. RAW 264.7 cells, cells were scraped and washed with ice-cold PBS at 4 °C. Proteins (30 μg/well) were fractionated by SDS-PAGE (10% (w/v) acrylamide, 0.275% (w/v) bisacrylamide gels) and electrotransferred at 100 V onto nitrocellulose membranes using a Trans-Blot® SD Semi-Dry Electrophoretic Transfer Cell (Bio-Rad, California, USA). Afterwards, membranes were stained with Ponceau Red, and then incubated for 30 min at room temperature with 5% Bovine Serum Albumin (BSA), to block nonspecific binding. Subsequently, the membranes were incubated overnight at 4 °C with primary antibodies at 1:1000 dilutions. The blots were incubated with species-specific HRP-conjugated secondary antibodies at 1:3000 in TBS-T with 5% BSA for 2 h at room temperature. Immunoreactivity was detected by enhanced chemiluminescence using Supersignal West Pico Chemiluminescent kit (Thermo Scientific). The chemiluminescence was captured with an ImageQuant LAS 4000 (GE Healthcare). The densitometric analyses were performed using Image-J® software. All results were expressed as relative ratio to β-actin, Lamin B or total protein content.

2.8. Phagocytosis assay

The phagocytic activity of macrophages stimulated with CAR and/or LPS was quantified by using *E. coli* fluorescent-labeled particles. Briefly, RAW 264.7 cells were pre-treated with CAR for 1 h and then treated for 24 h with LPS. At the end of treatments, phagocytosis was assessed according to Vybrant® phagocytosis Kit (Life Technologies). Fluorescence was quantified in a fluorescence spectrophotometer (i5 Softmax-Molecular Devices, USA) set at 480 nm/520 nm (excitation/emission).

2.9. Measurements of intracellular reactive species (RS) production (DCFH-DA oxidation)

Intracellular RS levels after 24 h of treatment with CAR 100 μM alone or in the presence of LPS (1 μg/mL) were measured by the DCFH-DA oxidation assay, as previously described [25]. This technique can be used as an index to quantify the overall RS production in cells. Briefly, after 24 h of treatment the culture medium was removed and 100 μM DCFH-DA dissolved in medium containing 1% FBS was added and incubated for 2 h at 37 °C in 5% CO_2 humidified air. After this period, the rate of DCFH oxidation was monitored for 30 min through kinetic reading of DCF fluorescence at 37 °C in a fluorescence plate reader (Spectra Max i3, Molecular Devices, USA) with an emission wavelength of 535 nm and an excitation wavelength of 485 nm. The results were expressed as percentage of DCF fluorescence rate in relation to control.

2.10. Measurements of nitrite levels

Quantification of the nitrite levels as an index of nitric oxide (NO) production was performed by Griess assay, as previously described [26]. The cells were pre-treated with CAR 100 μM for 1 h and then stimulated with LPS (1 μg/mL) for additional 24 h. The culture medium was collected and NO was indirectly detected by the quantification of its stable oxidative metabolite, nitrite [27]. Absorbance was assessed in a Spectra MAX i3 (Molecular Devices) spectrophotometer set at 540 nm.

2.11. Statistical analysis

The results were expressed as mean ± SEM. Data were evaluated by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons post-hoc test. The experiments were performed with n = 6 (*i.e.*, six wells per group). Data were analyzed by using GraphPad Prism software (San Diego, CA; version 7.00). Differences were considered to be significant when $^{**}p < 0.01$ $^{***}p < 0.001$, and $^{****}p < 0.0001$ in relation to control and $^{\#}p < 0.001$ and $^{\#\#}p < 0.0001$ in relation to LPS group.

3. Results

3.1. CAR supports macrophage survival against LPS

In order to evaluate the role of CAR in RAW 264.7 cells, we first performed a concentration curve to establish the growth inhibitory and effective dose concentrations (GI_{50}/ED_{50}) for CAR in MTT reduction and LDH activity assays, in order to use a non-cytotoxic dose in further experiments (Fig. 1A and B). At high doses, LPS becomes toxic to the cells, so we administered a concentration of 1 μg/mL in further experiments, which is capable of activating macrophages and inhibiting < 50% of their growth [26]. We decided to use a CAR concentration of 100 μM, which did not alter cell viability and has been previously reported to affect macrophage function [20]. CAR 100 μM did not cause toxicity to the cells, unlike LPS, which decreased cell viability. Pre-treatment with CAR was protective for the cells, since it was able to preserve MTT reduction and to inhibit LDH release stimulated by LPS (Fig. 1C and D).

3.2. CAR acts on ERK-dependent signaling pathway

The protein kinases ERK1/2, p38 and JNK are involved in the modulation of pro-inflammatory responses elicited by LPS during macrophage activation. We thus investigated the effect of CAR on LPS-induced phosphorylation of those kinases. Macrophages treated only with CAR did not present any changes in the phosphorylation levels of ERK1/2, p38 and JNK (Fig. 2A–C). Treatment with CAR 100 μM for 1 h prevented the effect of LPS (1 μg/mL, 15 min) in ERK1/2 phosphorylation (Fig. 2A). We did not observe any effect of CAR on p38 (Fig. 2B) and JNK (Fig. 2C) phosphorylation induced by LPS.

3.3. CAR impairs NF-κB nuclear translocation and activation induced by LPS

In order to evaluate the role of CAR in modulating the downstream targets of the ERK pathway, we evaluated NF-κB nuclear translocation and transcriptional activity. Macrophages were pre-treated for 1 h with CAR 100 μM and then exposed to LPS (1 μg/mL) for another hour. Cytosolic and nuclear fractions were fractionated and NF-κB immunocontent was evaluated by western blotting. LPS induced translocation of NF-κB from cytoplasm (Fig. 3A) to nucleus (Fig. 3B), an effect impaired by pre-treatment with CAR.

To verify the NF-kB activation, we evaluated its transcriptional activity by the luciferase-based assay. Cells were pre-treated with CAR for 1 h, followed by incubation with LPS overnight. CAR significantly inhibited NF-kB transactivation induced by LPS in RAW 264.7 cells (Fig. 3C).

3.4. CAR inhibits LPS-induced TNF-α and IL-1 β release but does not affect RAGE up-regulation

Since we observed a potential suppressive role of CAR on LPS-induced proinflammatory activation in RAW 264.7 cells, we then assessed the presence of pro-inflammatory cytokines in the extracellular medium. As shown in Fig. 4, cells treated with CAR 100 μM had no changes in TNF-α (Fig. 4A) and IL-1β levels (Fig. 4B). As expected, LPS induced a significant increase in the content of these cytokines, which was significantly inhibited by CAR. We also evaluated RAGE levels, since ERK1/2-mediated NF-kB activation is a major effect of the activation of this receptor in macrophages. However, no differences in RAGE levels were observed after 24 h of treatment.

3.5. CAR prevents phagocytosis and impairs LPS-stimulated RS and NO production

We next wondered whether CAR would be able to modulate macrophage physiology and activity. Therefore, we evaluated the processes of phagocytosis, production of RS and NO. LPS stimulated phagocytosis, which was prevented by pre-treatment with CAR 100 μM (Fig. 5A). Although the mean phagocytic activity in the DMSO group was lower than in LPS group, there is no statistical difference between these groups. On the other hand, there is a clear statistical difference between the LPS and the LPS + CAR group, evidencing an inhibitory effect by CAR. RS production was assessed by DCFH oxidation assay (Fig. 5B) and NO production was estimated by nitrite detection (Fig. 5C). LPS enhanced both RS and NO production, and CAR significantly inhibited these responses.

4. Discussion

CAR has been reported to exert antioxidant and anti-inflammatory actions *in vitro* and *in vivo* through a variety of mechanisms [15,18,19], but the molecular details of its actions are not well understood. Here, we described a protective role of CAR on RAW 264.7 macrophages against LPS-induced pro-inflammatory activation. LPS is a PAMP that is recognized by different receptors in macrophages and it triggers pro-inflammatory responses at cellular and systemic levels.

Stimulation of macrophages by LPS causes rapid cellular responses. Once recognized by pattern receptors, such as TLR4, CD14 and RAGE, LPS activates the phosphorylation of the mitogen-activated protein kinases ERK1/2, p38 and JNK [28]; here, only ERK1/2 activation was significantly inhibited by pre-treatment with CAR. Classically, the activation of such MAPKs by LPS binding to TLR4/CD14 complexes is mediated by different upstream modulators, such as Rac1 and Cdc42 (JNK), Ras/Raf (ERK 1/2) and ASK1/TAK1 (p38) [29,30]. This indicates that this action by CAR does not take place at extracellular level, or at least it does not interfere in LPS binding with TLR4/CD14. It is possible that CAR has a specific effect on Ras/Raf pathway, or that it acts on different membrane receptors that are also modulated by LPS.

Pro-inflammatory effects by LPS are also mediated by NF-kB-dependent transcriptional activation, and here we observed that the effects of LPS on NF-kB (p65) translocation from cytoplasm to nucleus and its luciferase-coupled transcription were blocked by CAR. We also observed the suppression of NF-kB transactivation by CAR even in the absence of LPS, which demonstrates its role as an inhibitor of the basal activation of NF-KB in RAW 264.7 macrophages. It is postulated that TLR4-mediated activation of NF-kB by LPS is dependent on TAK1, an upstream activator of both JNK and IKK [31], but our data demonstrated that CAR concomitantly inhibits ERK1/2 and NF-kB, indicating an alternate pathway.

In this context, ERK1/2 signaling plays a key role in inflammation,

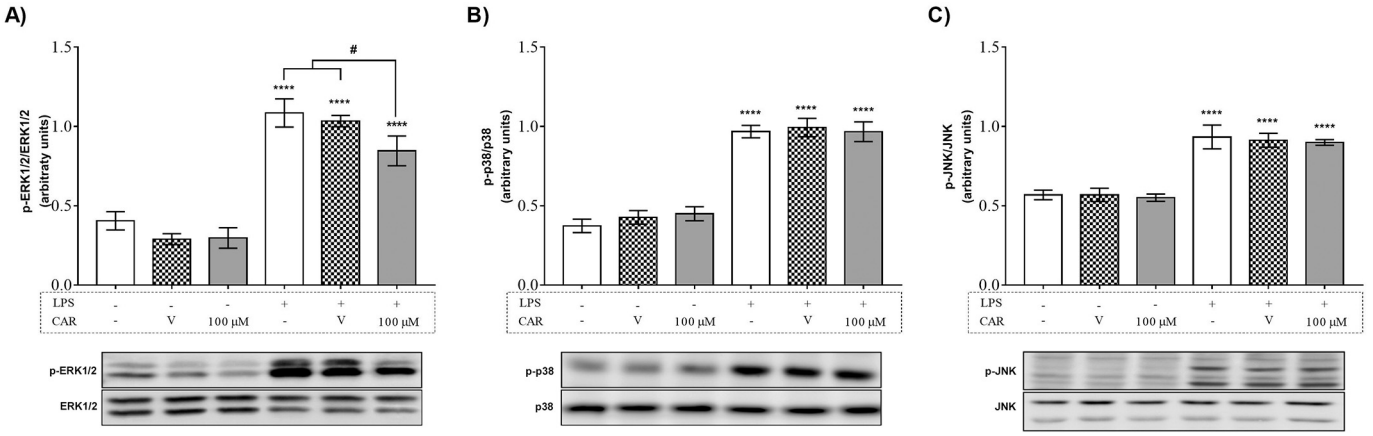

Fig. 2. Effect of CAR on ERK1/2, p38 and JNK phosphorylation. Cells were pre-treated with CAR for 60 min and then exposed to LPS for 15 min. Immunodetection of the phosphorylated and total isoforms of ERK1/2 (A), p38 (B) and JNK (C) was assessed by Western blotting. The results are expressed in arbitrary units of densitometric analysis. Statistical analysis (n = 3 in biological and experimental triplicate, one-way ANOVA followed by Tukey's) was performed: $^{****}p < 0.0001$ relative to control; $^{\#}p < 0\ 0.0001$ relative to LPS treated groups.

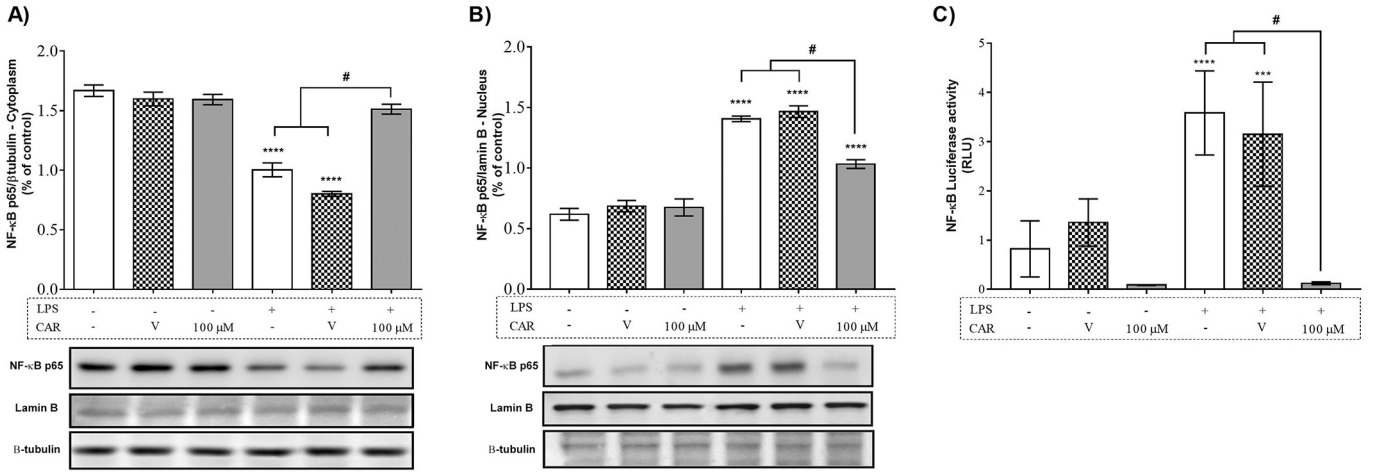

Fig. 3. Effect of CAR on LPS-induced NF-kB translocation to nucleus and transcriptional activation. RAW 264.7 macrophages were pre-treated for 1 h with CAR or vehicle and then challenged with LPS for 1 h to evaluate nuclear translocation of NF-kB from cytoplasm (A) to nucleus (B). The transcriptional activity of NF-κB was evaluated over a period of 12 h treatments by the reporter luciferase gene assay (C). Statistical analysis (n = 3 in biological and experimental triplicate, one-way ANOVA followed by Tukey's) was performed: $^{***}p < 0.001$, $^{****}p < 0.0001$ relative to control; $^{\#}p < 0.0001$, relative to LPS treated groups.

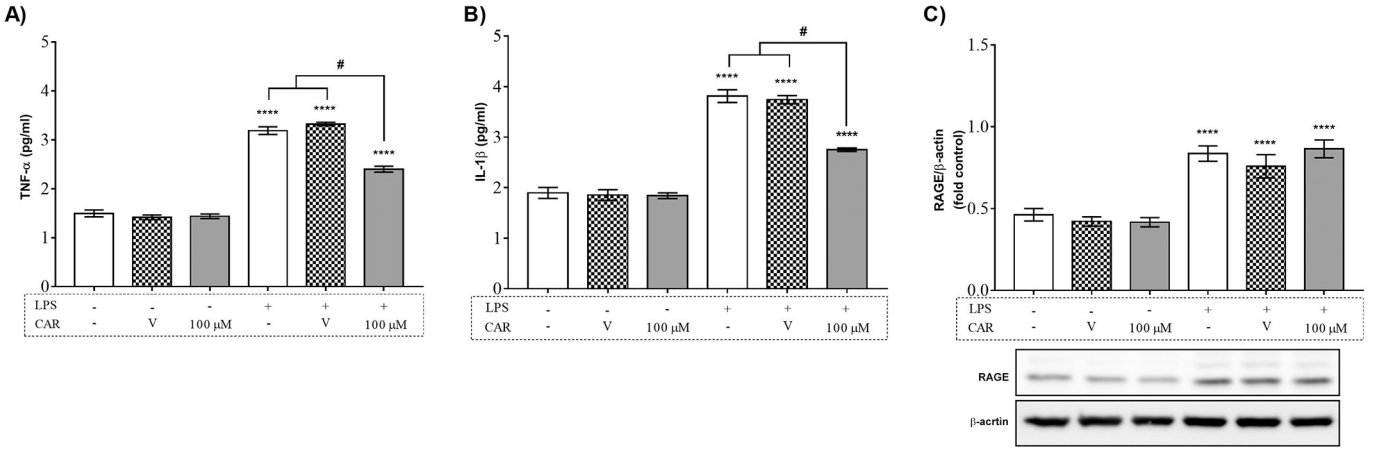

Fig. 4. Effect of CAR on TNF-α and IL-1β release and RAGE content in cells treated with LPS. RAW 264.7 macrophages pre-treated for 1 h with CAR or vehicle were subsequently challenged with LPS 1 μg/mL for 24 h. Secretion of pro-inflammatory cytokines TNF-α (A) and IL-1β (B) were evaluated by ELISA assay. The RAGE protein content (C) was assessed by Western blotting. Statistical analysis (ANOVA followed by Tukey's) was performed: $^{****}p < 0.0001$ relative to control; $^{\#}p < 0.0001$ relative to LPS treated groups.

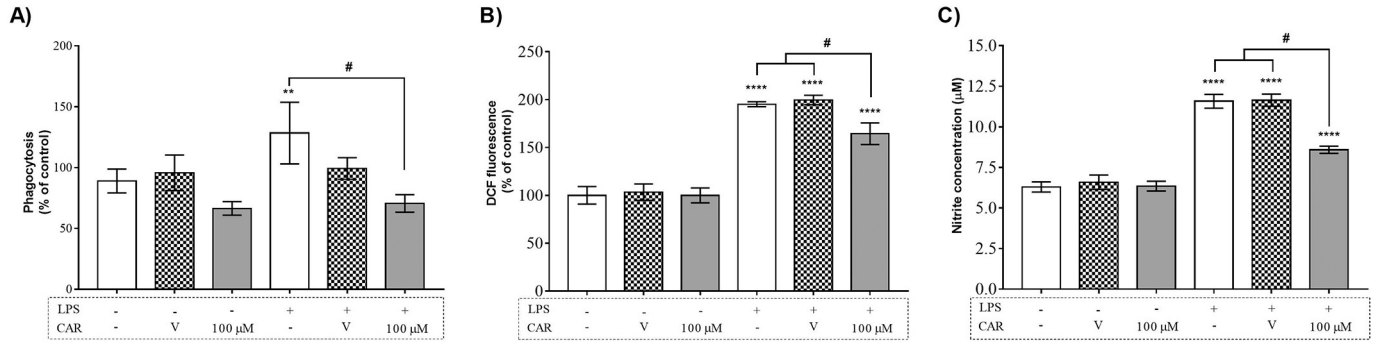

Fig. 5. Effect of CAR on phagocytic activation, RS production and nitrite accumulation induced LPS. RAW 264.7 macrophages pre-treated for 1 h with CAR or vehicle were subsequently challenged with LPS for 24 h. The phagocytic activity (A), RS production (B) and NO production (C) were measured at the end of incubation. Statistical analysis (n = 3 in biological and experimental triplicate, one-way ANOVA followed by Tukey's) was performed: $^{**}p < 0.01$,$^{****}p < 0.0001$ relative to control; $^{\#}p < 0.001$ relative to LPS treated groups.

not only through activation of downstream factors such as NF-kB, but also as a key factor during inflammatory burst, resulting in phagocytosis activation. Previous study shows that inhibition of ERK is able to negatively regulate phagocytosis [32], which is in accordance with our results, since the induction of ERK1/2 phosphorylation and NF-kB activation by LPS and subsequent phagocytosis stimulation were inhibited by CAR. Those authors also described the link between ERK1/2 and mitochondria, and suggest that the decrease in phagocytosis observed with ERK inhibition may be a partial consequence of the loss of ATP, considering the high energy needs of the phagocytic process. This could explain the mechanism by which CAR protects against LPS-induced RAW 264.7 cell death at 100 µM, since CAR has been shown to provide protection against apoptosis in many injuries [33–35]. However, CAR exhibits pro-apoptotic effects in multiple cancer cell lines [36], supporting the notion that the effects of CAR are cell type-dependent. Here, CAR protected macrophages against LPS-induced inflammation and death, suggesting an anti-apoptotic effect, which may be further studied in the near future; this opens perspectives to explore novel potential applications for CAR in pharmacological research.

Activated macrophages generate RS which are crucial to abolish foreign insults; nonetheless, RS also participate in other physiological processes, including cell signaling and proliferation. The pro-inflammatory effects of LPS analyzed here (ERK1/2 and NF-kB activation, NO production and activation of phagocytosis) are all associated to increased RS production. During macrophage activation, RS are mainly generated by NADPH oxidase activation during phagocytosis, and the direct interaction of TLR4 with NADPH oxidase subunit Nox4 is involved in LPS-mediated RS generation and NF-κB activation [37]. NADPH oxidase-mediated RS production is also activated in response to RAGE and it is associated to NF-kB activation [38]. Previous studies demonstrate that CAR has strong antioxidant activity in different models [15]. As the production of RS is directly related to the activation of a pro-inflammatory state in the cell, antioxidant proprieties of CAR could explain most of the anti-inflammatory effects here.

In a previous work, RAGE silencing selectively inhibited ERK1/2 and NF-kB activation in response to extracellular HSP70 (which acts as a DAMP in the extracellular environment), while p38 and JNK activation were not affected [24]. This could suggest that CAR acts on the RAGE/ERK/NF-kB pathway, but data on the effect of CAR on RAGE content did not confirm this hypothesis. It is known that agonist binding to RAGE triggers its up-regulation, since the *AGER* gene has a NF-kB responsive element in the proximal promoter, while inhibition of RAGE blocks its own expression [39]. Here, RAGE levels were increased in response to LPS, and since RAGE was previously described to be activated by LPS [2,40], we measured the expression of RAGE after prolonged exposition to LPS and CAR. However, pre-treatment with CAR 100 µM did not affect LPS-induced RAGE up-regulation, indicating that the inhibitory effect on ERK1/2 and NF-kB was not mediated by this receptor. It is possible that CAR is not acting on a specific molecular target such as RAGE or another upstream molecule, but through an antioxidant mechanism that affects redox-sensitive domains in specific proteins. Proteins such as ERK1/2 and NF-kB isoforms are subject to redox regulation [41,42] and our data demonstrating an antioxidant effect of CAR are suggestive that this could be the inhibitory mechanism of CAR over ERK/NF-kB pathway. It is possible, also, that redundant transcription factors are responsible for RAGE up-regulation, such as Erg1 [43] and SP-1 [44].

Together, our results add relevant information on the cellular mechanisms by which CAR protects RAW 264.7 macrophages against LPS-induced pro-inflammatory activation. This flavonoid was able to reduce the activation of ERK1/2 phosphorylation, NF-kB p65 translocation to nucleus and transcriptional transactivation, phagocytic activation and IL-1β and TNF-α release. The effects of CAR do not seem to be mediated by inhibition of specific membrane receptors that are activated by LPS, such as TLR4 or RAGE; on the other hand, the inhibition of DCFH-sensitive RS production and nitrite accumulation indicate that the antioxidant properties of CAR are key to its anti-inflammatory effect at cellular level.

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Authors' contributions

NS and TKR carried out experiments, analyzed the data and drafted the manuscript. FZC and MSM contributed to perform for enzyme-linked immunosorbent assay (ELISA). AGG, LJQJ and JCFM performed data evaluation. DPG supervised and coordinated this work. All authors read and approved the final manuscript.

Declaration of Competing Interest

The authors declare no conflicts of interest.

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