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ORIGINAL ARTICLE

Capsaicin inhibits lipopolysaccharide-induced adrenal steroidogenesis by raising intracellular calcium levels

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Abstract

Introduction Glucocorticoid release by adrenals has been described as significant to survive sepsis. The activation of transient receptor potential vanilloid type 1 (TRPV1) inhibited ACTH-induced glucocorticoid release by adrenal glands in vitro.

Objective The aim of this study was to investigate if capsaicin, an activator of TRPV1, would prevent LPS-induced glucocorticoid production by adrenals.

Methods Male Swiss-Webster mice were treated with capsaicin intraperitoneally (0.2 or 2 mg/kg) 30 min before LPS injection. All analyses were performed 2 h after the LPS stimulation, including plasma corticosterone and peritoneal IL-1β and TNF-α levels. Furthermore, murine adrenocortical Y1 cells were used to assess the effects of capsaicin on LPS-induced corticosterone production in vitro.

Results Capsaicin (2 mg/kg, i.p.) significantly reduced plasma corticosterone levels and adrenal hypertrophy induced by LPS without alter the levels of pro-steroidogenic cytokines IL-1β and TNF-α in peritoneal cavity of mice, while the dose of 0.2 mg/kg of capsaicin did not interfere with adrenal steroidogenesis, attested by RIA and ELISA, respectively. Y1 cells express TRPV1, measured by immunofluorescence and western blot, and capsaicin decreased LPS-induced corticosterone production by these cells in vitro. Capsaicin also induces calcium mobilization in Y1 cells in vitro.

Conclusions These findings suggest that capsaicin inhibits corticosterone production induced by LPS by acting directly on adrenal cells producing glucocorticoids, in a mechanism probably associated with induction of a cytoplasmic calcium increase in these cells.

Keywords Calcium · Capsaicin · Glucocorticoid · LPS · Steroidogenesis · TRPV1

Introduction

Rapid cortisol production by adrenals has been reported as important to survive sepsis [1, 2]. Although elevated cortisol levels have been diagnosed in the early time period after admission of patients with severe sepsis to the intensive care units, low plasma adrenocorticotropic hormone (ACTH) levels were reported [3, 4]. The activation of adrenal steroidogenesis in sepsis has been attributed to factors independent of classical ACTH activation, including pro-steroidogenic cytokines, such as interleukin (IL)-1β, IL-6, tumor necrosis factor (TNF)-α, prostaglandins, and lipopolysaccharide (LPS)-induced TLR-signaling [5, 6].

Transient receptor potential vanilloid type 1 (TRPV1) is a ligand-gated nonselective cation channel with high permeability to Ca^{2+} [7], mainly expressed in primary sensory neurons of the dorsal root and trigeminal ganglia of the

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peripheral nervous system [8]. TRPV1 is also present in the adrenal gland and its expression is elevated in stress situation or when these glands are incubated with ACTH [9], suggesting a role of TRPV1 in modulating glucocorticoid production. TRPV1 is a polymodal receptor activated by physical and chemical stimulation, including heat (above 43 °C) and changes in pH (both acidic and alkaline), endovanilloids, such as anandamine, N-acyldopamines, and a variety of exogenous agonists, which mainly consist of capsaicin, the pungent component of Chili pepper [7, 10].

Glucocorticoids produced during sepsis play a key role in ensuring proper cardiovascular reactivity to angiotensin II and catecholamines, and to control overactivation of the immune system [11, 12]. However, high and sustained glucocorticoid levels can be deleterious to cells and tissues. Hypercorticoidism can induce harmful effects on the central nervous system (CNS), including neuroinflammation, loss of neuronal function, and apoptosis of neuronal cells, triggering a reduction in hippocampal neurogenesis and an increase in neurodegeneration [13–15]. Bearing in mind that LPS-induced CNS inflammation and neurodegeneration was independent of systemic pro-steroidogenic cytokines, including IL-1β and IL-6 [16], the inhibition of LPS-induced adrenal steroidogenesis may be an interesting strategy to prevent the development of CNS disorders in patients with severe sepsis. Considering that anandamine inhibited ACTH-induced corticosterone release by adrenal glands in vitro by a mechanism dependent of TRPV1 [9], we undertook this study to evaluate if capsaicin, a TRPV1 activator, can prevent glucocorticoid production induced by LPS.

Materials and methods

Chemicals

Adenosine triphosphate (ATP), capsaicin, and LPS were purchased from Sigma Chemical Co. (Saint Louis, MO, USA), and sodium heparin from Roche (São Paulo, SP, Brazil). All solutions were freshly prepared immediately before use.

Animals and treatments

Male Swiss-Webster mice (4–6 weeks old) were obtained from the Oswaldo Cruz Foundation breeding colony and used in accordance with the guidelines of the Committee on Use of Laboratory Animals of the Oswaldo Cruz Foundation (CEUA-FIOCRUZ, license L-027/2016). Mice were housed in groups of four in a temperature-, humidity-, and light-controlled (12 h light: 12 h darkness cycle) colony room. Mice were given ad libitum access to food and water. Thirty-two male mice were randomly assigned into four groups as follows: control ($n = 8$); stimulated with LPS ($n = 8$); treated with capsaicin 0.2 mg/kg and stimulated with LPS ($n = 8$); treated with capsaicin 2 mg/kg and stimulated with LPS ($n = 8$). The mice were treated with capsaicin intraperitoneally 30 min before challenge with LPS (100 ng/cavity, i.p.). The analyses were performed 2 h after challenge with LPS.

Cytokine evaluation

Two hours after LPS challenge, the peritoneal lavage recovered was centrifuged (400 × g, 10 min, 4 °C) and the supernatant immediately frozen in liquid nitrogen, and stored at −80 °C. IL-1β and TNF-α were assayed in the peritoneal lavage using commercial ELISA kit according to the manufacturer's instructions (R&D Systems, Minneapolis, MN, USA).

Cell culture

We used immortalized adrenocortical Y1 cells, which is an adrenal cortex tumor cell line of a male LAF1 mouse produced by steroid hormones, including corticosterone. Cells were used until the 20th passage. Y1 cells were maintained in Dulbecco's modified Eagle's medium/Nutrient mix F12K (DEMEM/F12K). All culture mediums were supplemented with 2.5% fetal bovine serum (FBS), 15% horse serum, and 4 mM L-glutamine in a humidified atmosphere containing 5% CO_2: 95% air atmosphere at 37 °C.

Immunocytochemistry

Y1 cells were fixed in 4% formaldehyde at room temperature for 10 min. After permeabilization in 0.05% Triton X-100 for 30 min at room temperature, slides were washed in phosphate-buffered saline (PBS). The unspecific sites were blocked with 3% bovine serum albumin (BSA) for 10 min. The cells were incubated overnight with rabbit polyclonal antibody anti-TRPV1 (R-130; Santa Cruz Biotechnology, Dallas, TX, USA) and then with Alexa 488 anti-goat (Molecular Probes, Eugene, OR, USA) for 2 h. Next, they were washed several times with PBS, mounted with an aqueous mounting medium with DAPI (Prolong Gold antifade reagent; Life Technologies, Carlsbad, CA, USA). Slides were viewed by both phase-contrast and fluorescence microscopy, and digital images were obtained using a DP72 Olympus digital camera with the Olympus micro imaging software cellSens platform (Olympus Corp., Tokyo, Japan).

Western blot

Y1 cells (10^6 cells) were homogenized in cold lysis buffer containing the complete protease inhibitor cocktail

(F. Hoffmann-La Roche Ltd.) and 0.1% Triton X-100 in PBS. The lysate was centrifuged at 13,000 × *g* for 10 min at 4 °C. The supernatant was recovered and the proteins were quantified using a BCA assay according to the manufacturer's instructions (Sigma–Aldrich Corp.). Equal amounts of protein (50 µg/lane) were loaded and separated on a 12.5% SDS–PAGE gel, and transferred to nitrocellulose membranes (GE Healthcare, Little Chalfont, UK). After blocking in 5% (w/v) skimmed milk powder in TBS-T for 1 h, the membranes were exposed overnight at 4 °C to TRPV1-specific rabbit polyclonal antibodies (1:100; Santa Cruz Biotechnology), or a GAPDH-specific goat monoclonal antibody (MAB) (1:1000; Santa Cruz Biotechnology), followed by three washes and incubation with an IRDye goat anti-mouse secondary antibody (1:10 000; LI-COR Corporate, Lincoln, NE, USA) for 1 h at room temperature. The membranes were washed in TBS-T, and protein expression was detected using fluorescence with the Odyssey Image System (LI-COR Corporate).

Corticosterone quantification

Animals were euthanized in a CO_2 chamber, during the nadir (08:00 h) of the circadian rhythm, as described previously [17, 18], and blood was immediately collected from cardiac puncture with heparinized (400 U/ml) saline. Plasma was obtained after sample centrifugation for 10 min at 1000 × *g* and stored at −20 °C until use. In in vitro assays, Y1 cells (5×10^5 cells/well) were treated with capsaicin (1–25 µM) at 37 °C for 30 min and then stimulated with LPS (10 µg/ml) in vitro for 24 h at 37 °C in 5% CO_2:95% air atmosphere. After centrifugation at 150 × *g* for 10 min, the supernatant was collected and stored at –20 °C until use. The plasma and supernatant of cell culture corticosterone levels were detected by radioimmunoassay (RIA), following the manufacturer's guidelines (MP Biomedicals, Solon, OH, USA).

Single-cell calcium imaging

Variations of free intracellular calcium levels ($[Ca^{2+}]_i$) were evaluated in single cells obtained from adrenocortical Y1 cell culture by following an adaptation of the protocol as described previously [19]. Forty-eight hours before the assay, Y1 cells were placed in a 15-mm coverslip. The adrenocortical Y1 cells were loaded for 40 min with 5 µM Fura-2/AM (molecular probes), 0.1% fatty acid-free BSA, and 0.02% pluronic acid F-127 (Molecular Probes) in Krebs solution (132 mM NaCl, 4 mM KCl, 1.4 mM $MgCl_2$, 2.5 mM $CaCl_2$, 6 mM glucose, 10 mM HEPES, pH 7.4), at 37 °C in 5% CO_2:95% air atmosphere. After a 10-min post-loading period at room temperature in Krebs solution, to obtain a complete hydrolysis of the probe, a 15-mm coverslip with the cells was mounted on a chamber in a PH3 platform (Warner Instruments, Hamden, CT, USA) on the stage of an inverted fluorescence microscope (Axiovert 200, Carl Zeiss, Oberkochen, Germany). Cells were continuously perfused with KS and stimulated with different solutions. The variations in $[Ca^{2+}]$i were evaluated by quantifying the ratio of the fluorescence emitted at 510 nm following alternate excitation (750 ms) at 340 and 380 nm, using a Lambda DG4 apparatus (Sutter Instrument, Novato, CA, USA) and a 510-nm long-pass filter (Carl Zeiss) before fluorescence acquisition using a ×40 objective and a Cool SNAP digital camera (Roper Scientific, Trenton, NJ, USA). Eighty cells were analyzed per coverslip. Acquired values were processed using MetaFluor software (Universal Imaging Corp., West Chester, PA, USA). Values for Fura-2 fluorescence ratio were calculated based on a cut-off of 10% increase in the $[Ca^{2+}]$i level induced by the stimulus.

Statistical analysis

The data are reported as the mean ± standard error of the mean (SEM). All data were analyzed in a double-blinded manner and evaluated to ensure normal distribution. Statistical analysis was performed with one-way ANOVA followed by the Newman–Keuls–Student multi comparison test. Probability values (p) of 0.05 or less were considered statistically significant.

Results

Capsaicin inhibits LPS-induced hypercorticoidism independent of altered levels of pro-steroidogenic cytokines TNF-α and IL-1β produced in vivo

We noted that an intraperitoneal dose of LPS induced adrenal hypertrophy 2 h after injection, as evidenced by the ratio between adrenal weight (mg) and body weight (g). Treatment with capsaicin prevented LPS-induced increase in adrenal size at 2 mg/kg given intraperitoneally, being inactive at 0.2 mg/kg (Fig. 1a). The absolute adrenal weights shown in Fig. 1a are 0.9 ± 0.1, 2 ± 0.1, 2.1 ± 0.2, and 0.9 ± 0.05 mg (mean ± SEM) for control mice, LPS-provoked mice, LPS-provoked mice treated with capsaicin 0.2 mg/kg, and LPS-provoked mice treated with capsaicin 2 mg/kg, respectively. In parallel, we showed that mice stimulated with LPS presented a significant increase in plasma corticosterone levels compared to controls. Treatment with capsaicin significantly impaired the increase in plasma corticosterone levels induced by LPS at 2 mg/kg given intraperitoneally, but it was not modified at 0.2 mg/kg (Fig. 1b).

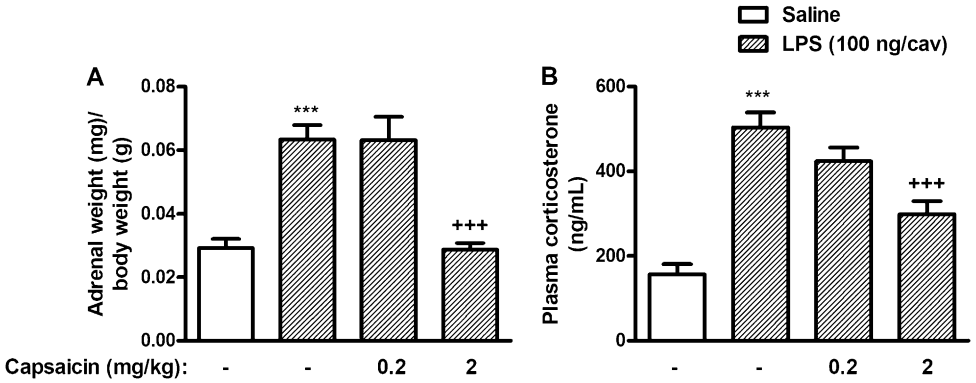

Fig. 1 Capsaicin inhibits LPS-induced adrenal hypertrophy and hypercorticoidism in vivo. Capsaicin inhibited LPS-induced adrenal hypertrophy **a** and plasma corticosterone levels **b**. Each value represents the mean ± S.E.M. The statistical analysis was performed by ANOVA followed by Newman–Keuls–Student's test. $^{***}p < 0.001$ compared to the saline-provoked group. $^{+++}p < 0.001$ compared to the LPS-provoked group

Next, we evaluated whether inhibition of glucocorticoid production by capsaicin was related to a reduction in pro-steroidogenic cytokines. We observed that LPS induced a rise of TNF-α and IL-1β levels in the peritoneal cavity of mice 2 h after injection (Fig. 2a, b, respectively). Treatment with capsaicin increased LPS-induced TNF-α at 0.2 mg/kg, being inactive at 2 mg/kg (Fig. 2a). Nonetheless, capsaicin did not interfere with LPS-induced IL-1β at any of the doses used (Fig. 2b).

Capsaicin inhibits LPS-induced production of corticosterone and induces high percentage of calcium influx in Y1 cells that express TRPV1 in vitro

In order to evaluate whether capsaicin can act directly on adrenocortical cells in vitro, we investigated if Y1 cells express TRPV1. Y1 cells express TRPV1 in the cytoplasm of the cells in a diffuse manner, attested by immunofluorescence (Fig. 3a–f). The expression of TRPV1 in Y1 cells was confirmed by western blot (Fig. 3g). Then, we analyzed the effect of capsaicin, an activator of TRPV1, on corticosterone production by Y1 cells in vitro. Although capsaicin did not alter the corticosterone production by Y1 cells at any of the concentrations used, it inhibited LPS-induced corticosterone release by Y1 cells in vitro at all concentrations, the most prominent inhibitory effect being at 25 μM (Fig. 4a).

Since activation of TRPV1 leads to an increase in intracellular calcium, important both for the release of glucocorticoids and for the inhibition of its production, we evaluated the effect of capsaicin on the intracellular calcium levels. As expected from an inductor of steroidogenesis, LPS increased intracellular calcium levels in Y1 cells in vitro. Capsaicin induced an increase in calcium influx in Y1 cells in vitro more prominently compared to that observed with the LPS stimulus. ATP (1 mM) application served as a positive control for cell viability (Fig. 4b).

Discussion

This work investigated the effects of capsaicin on LPS-induced steroidogenesis in the adrenal gland. Our results revealed that capsaicin inhibited LPS-induced corticosterone production by adrenocortical cells in vivo and in vitro. However, capsaicin did not reduce the secretion of pro-steroidogenic cytokines IL-1β and TNF-α induced by LPS in the peritoneal cavity of mice. The anti-steroidogenic activity of capsaicin appeared to be associated with activation of TRPV1 and induction of high amounts of calcium influx in adrenocortical cells directly. These findings suggest that capsaicin (and other TRPV1 activators) is indeed a promising therapeutic strategy to control diseases related to hypercorticoidism, including some systemic bacterial infections.

In this study, we showed that capsaicin inhibited LPS-induced increase of both circulating levels of corticosterone and adrenal hypertrophy in mice. These data suggest that capsaicin decreases the production of steroidogenic machinery in adrenal glands, since we and others showed that adrenal hypertrophy is directly related to the presence of high expression of steroidogenic enzymes, including 11β-hydroxysteroid dehydrogenase-type 1 (11β-HSD1) and steroidogenic acute regulatory protein (StAR) [17, 20]. Furthermore, LPS-induced glucocorticoid production depends on elevated expression of StAR [21]. In infectious diseases, including sepsis, the steroidogenesis in adrenal glands can be modulated in an ACTH-independent fashion, by cytokines such as IL-1β, IL-6, and TNF-α [4–6, 21]. We observed that capsaicin did not reduce the production of pro-steroidogenic cytokines IL-1β and TNF-α induced by

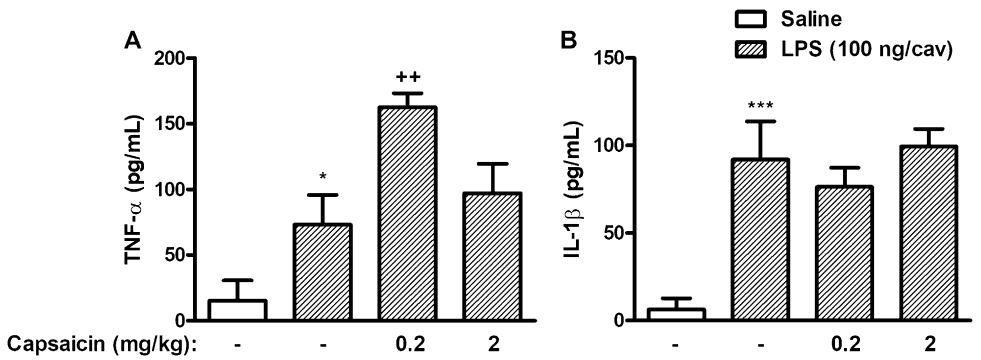

Fig. 2 Capsaicin increases LPS-induced peritoneal TNF-α without altering IL-1β production in vivo. Effect of capsaicin on increased TNF-α **a** and IL-1β **b** levels in the peritoneal cavity of mice induced by LPS. Each value represents the mean ± SEM. The statistical analysis was performed by ANOVA followed by Newman–Keuls–Student's test. $^{*}p < 0.05$ compared to the saline-provoked group. $^{***}p < 0.001$ compared to the saline-provoked group. $^{++}p < 0.01$ compared to the LPS-provoked group

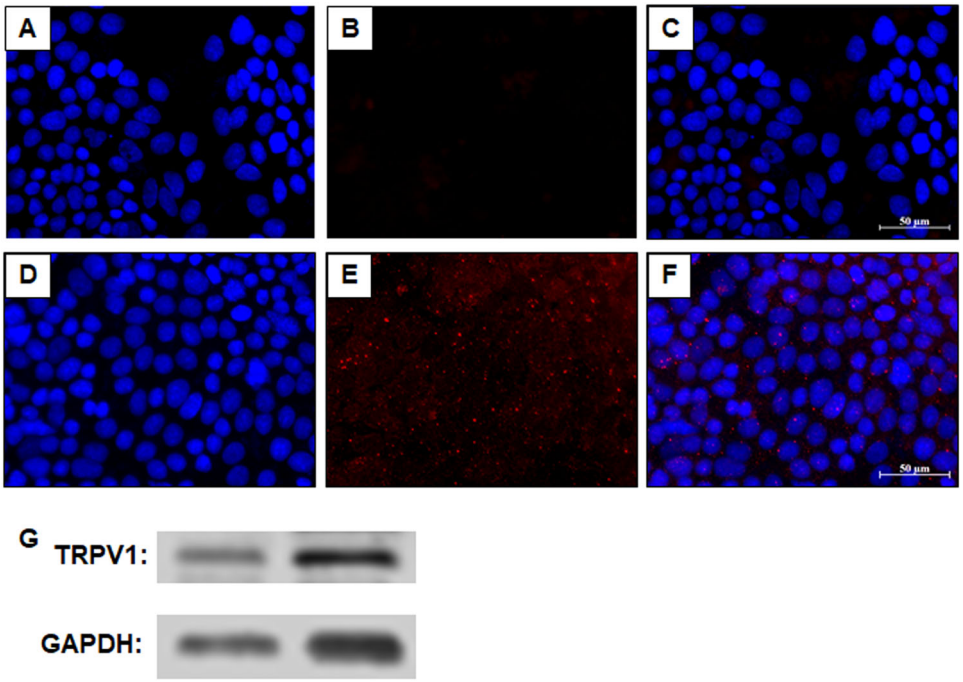

Fig. 3 Adrenocortical Y1 cells express TRPV1. TRPV1 was expressed in the cytoplasm in all Y1 cells evaluated. Immunolocalization of TRPV1 **e** in Y1 cells and negative controls **b**. **a**, **d** Sample images of DAPI-stained cells. **c** Merged image of panels a and b. **f** Merged image of panels d and e. **g** Expression of TRPV1 in Y1 cells was determined by western blot. Each lane represents a lysate of Y1 cells in different passages

LPS. In addition, although systemic inflammation has significant deleterious effects on the progression of neurodegenerative diseases, LPS-induced CNS inflammation and neurodegeneration are independent of circulating IL-1β and IL-6 levels [16]. Altogether, these data are in line with our hypothesis that glucocorticoids may be important for the neurodegeneration observed in sepsis. The fact that capsaicin had no effect on the production of cytokines suggests that capsaicin-inhibited glucocorticoid synthesis is related to a direct action in adrenocortical cells.

In order to verify whether capsaicin acts directly in adrenocortical cells, we used immortalized adrenocortical Y1 cells. In fact, LPS induced glucocorticoid production directly in adrenocortical cells by a mechanism that depends on the activation of TLR4 and NFκB [5, 22]. First, Y1 cell was shown to express TRPV1, which is the receptor to capsaicin. These data are in agreement with the literature as TRPV1 is expressed in the adrenal gland [9]. Then, we showed that capsaicin inhibited LPS-induced corticosterone secretion by Y1 cells in vitro. Our data suggest that capsaicin inhibited steroidogenesis in adrenocortical cells by activation of TRPV1, once Y1 cells express TRPV1 and the activation of its receptor reduced ACTH-induced corticosterone release by the adrenal gland [9].

In an attempt to clarify how capsaicin decreases the production of glucocorticoids by Y1 cells, we evaluated

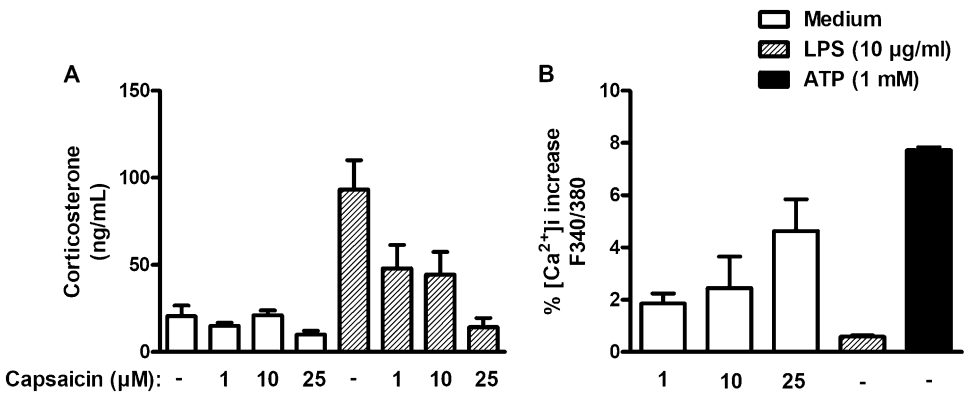

Fig. 4 Capsaicin inhibits LPS-induced production of corticosterone and induces calcium influx in Y1 cells in vitro. **a** Capsaicin blocked the LPS-induced production of corticosterone by Y1 cells in vitro. **b** Capsaicin increased the LPS-induced calcium influx in Y1 cells in vitro. Each value represents the mean ± SD of triplicate determinations from a single experiment and is representative of three experiments

calcium influx in these adrenocortical cells, since TRPV1 is a ligand-gated nonselective cation channel with high permeability to Ca^{2+} [7]. We noted that capsaicin induced a higher intracellular calcium influx in Y1 cells compared to LPS stimulus in vitro. Although intracellular calcium is important for the production and secretion of glucocorticoids by adrenocortical cells, it was shown that high levels of intracellular calcium do the opposite, i.e. inhibit the production of glucocorticoids [23]. Therefore, our data suggest that inhibition of capsaicin-induced glucocorticoid production in adrenocortical cells is related to elevated intracellular calcium levels.

The hypothalamus-pituitary-adrenal (HPA) axis is the main neuroendocrine system that controls responses to stress, as in pathological conditions, including infectious diseases [24]. In sepsis, a very fast glucocorticoid release, which is the major product of HPA axis, is important for patient survival [1, 25]. Currently, a significant decrease in the mortality rates of septic patients has been noted in developed countries [26]; however, many survivors who return home after hospitalization present a dramatic reduction in their functional capacities and quality of life, with cognitive decline (the loss of memory) and executive function more frequently affected [27]. High and sustained glucocorticoid levels induce deleterious effects on the CNS, with reduction in hippocampal neurogenesis and a rise in neurodegeneration [13, 14]. In this sense, we believe that hypercorticoidism might be related to the cognitive decline observed in septic patients. Therefore, discovery of potential modulators of LPS-induced endogenous glucocorticoid production, especially activators of TRPV1, may be important for the development of promising future therapeutic targets in sepsis. In summary, our results indicate that capsaicin inhibits LPS-induced steroidogenesis in adrenocortical cells by a mechanism apparently related to activation of TRPV1 and increase in intracellular calcium levels.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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