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Anti-inflammatory and analgesic effects of the sesquiterpene lactone budlein A in mice: Inhibition of cytokine production-dependent mechanism

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Abstract

The anti-inflammatory activities of some medicinal plants are attributed to their contents of sesquiterpene lactones. In the present study, the anti-inflammatory and anti-nociceptive activity of a sesquiterpene lactone isolated from *Viguiera robusta*, budlein A in mice was investigated. The treatment with budlein A dose—(1.0–10.0 mg/kg, p.o., respectively) dependently inhibited the carrageenan-induced: i. neutrophil migration to the peritoneal cavity (2–52%), ii. neutrophil migration to the paw skin tissue (32–74%), iii. paw oedema (13–74%) and iv. mechanical hypernociception (2–58%) as well as the acetic acid-induced writhings (0–66%). Additionally, budlein A (10.0 mg/kg) treatment inhibited the mechanical hypernociception-induced by tumour necrosis factor (TNF-α, 36%), Keratinocyte-derived chemokine (KC, 37%) and Interleukin-1β (IL-1β, 28%), but not of prostaglandin E_2 or dopamine. Budlein A also inhibited the carrageenan-induced release of TNF-α (52%), KC (70%) and IL-1β (59%). Furthermore, an 8 days treatment with budlein A inhibited Complete Freund's adjuvant (10 μl/paw)-induced hypernociception, paw oedema and paw skin myeloperoxidase activity increase while not affecting the motor performance or myeloperoxidase activity in the stomach. Concluding, the present data suggest that budlein A presents anti-inflammatory and antinociceptive property in mice by a mechanism dependent on inhibition of cytokines production. It supports the potential beneficial effect of orally administered budlein A in inflammatory diseases involving cytokine-mediated nociception, oedema and neutrophil migration.

Keywords: Budlein A; *Viguiera robusta*; Asteraceae; Sesquiterpene lactones; Inflammation; Oedema; Nociception; Hypernociception; Hyperalgesia; Neutrophil migration; Pain; Cytokine; Chemokine

1. Introduction

The pharmacological activities of some medicinal plants, specially those from the sunflower family Asteraceae, are attributed to their contents of sesquiterpene lactones such as mikanolide, helenalin, parthenolide, artemisinin, bis(isoalantodiol-B)glutarate. In fact, sesquiterpene lactones may present a wide variety of activities including *in vitro* antimicrobial (Pickman, 1984), antiviral (Meshnick, 2002), and antitumor activities (Chen et al., 1994). Sesquiterpene lactones also seem promising anti-inflammatory drugs. They inhibit inflammatory oedema induced by cotton pellet granuloma, complete Freund's adjuvant, 4-beta-phorbol 12-myristate 13-acetate, formalin, and carrageenan (Damre et al., 2003; Guardia et al., 2003; Abil'daeva et al., 2004; Silvan et al., 1996; Feltenstein et al., 2004). Additionally, using the acetic acid-induced writhings model it was demonstrated the antinociceptive effect of sesquiterpene lactones (*e.g.* parthenolide, costunolide, dehydrocostus lactone) (Jain and Kulkarni, 1999; Okugawa et al., 2000; Ahmed et al., 2001). Furthermore, Recio et al. (2000), demonstrated the concomitant inhibition by different sesquiterpene

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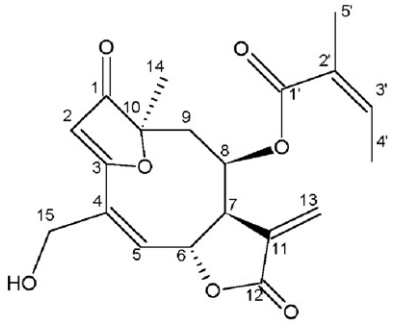

Fig. 1. Chemical structure of budlein A.

lactones (*e.g.* confertdiolide) of inflammatory oedema and leukocyte migration to ear skin challenged with 12-*O*-tetradecanoylphorbol 13-acetate.

Budlein A (Fig. 1) is a sesquiterpene lactone that has been previously isolated from *Viguiera buddleiaeformis* (De Vivar et al., 1976). However, despite its first isolation in the seventies, there are only two reports regarding the budlein A effect on mammalian cells, which demonstrate *in vitro* inhibition of sperm motility (Huacuja et al., 1993) and NF–kB activation (Siedle et al., 2004). Inhibition of NF–kB activity by preventing I–kB degradation has been described for other sesquiterpene lactones including isogoyazensolide, centratherin, atripliciolide tiglate, among others (Hehner et al., 1998; Siedle et al., 2004). The activation of this transcription factor is involved in the production of many inflammatory mediators. After its activation, NF–kB migrates to the cell nucleus and induces the expression of cytokines, such as tumour necrosis factor (TNF-α), interleukin-1β (IL-1β), interleukin-6 (IL-6), cyclooxigenase-2 and adhesion molecules (L-selectins, ICAM-1) (Baeuerle and Henkel, 1994; Baeuerle and Baltimore, 1996; May and Ghosh, 1998; for review see Barnes, 2006), which are important for the genesis of inflammatory signals.

Thus, in the present study we isolated budlein A from the diclorometanic extract of *Viguiera robusta*, and demonstrated the anti-inflammatory and anti-nociceptive effects of budlein A in models of carrageenan-induced oedema, leukocyte migration and nociception. Furthermore, the mechanisms involved in budlein A effect were also addressed.

2. Materials and methods

2.1. Isolation of budlein A

2.1.1. Plant material

Leaves of *V. robusta* were collected by F.B.C. in April 2001 in Batatais (35 km, Batatais–Altinopolis highway), state of São Paulo, Brazil. E. E. Schilling (Department of Botany, University of Tennessee, Knoxville, TN, USA) and J. N. Nakajima (Biology Institute, University of Uberlândia, Uberlândia, MG, Brazil) identified the material. A voucher specimen (FBC # 105) is deposited at the Herbarium SPFR of the Department of Biology, FFCLRP, University of São Paulo, Ribeirão Preto, SP, Brazil, with the code SPFR 07155.

2.1.2. Extraction and isolation

Air-dried and entire leaves (2.5 kg) of *V. robusta* were placed in an Erlenmeyer and extracted with dichloromethane in sonicator, at room temperature (28 °C), for 10 min. The residue was filtered through common filter paper and the solvent was removed under vacuum, affording 14 g of dried crude extract, which was analyzed by infrared (IR) spectroscopy. A strong band at 1.760 cm^{-1} in the spectrum corresponded to the carbonyl stretching of γ-lactones, an indication of sesquiterpene lactones in the extract. In order to remove pigments and fats, the extract was dissolved in methanol-water (4:1) and successive partition was made with *n*-hexane, dichloromethane and methanol, affording, respectively 3.1, 4.0, and 6.5 g of organic soluble residues after solvent evaporation under vacuum. After IR spectral analysis, a strong band of γ-lactones was observed in the spectrum of the dichloromethane residue. This residue was fractioned through vacuum liquid chromatography (silica gel, Merck, *n*-hexane: ethyl acetate, increasing polarity) to give nine fractions after thin-layer chromatography (TLC) analysis. Fraction 6 (1042 mg) and 7 (725 mg) were found to contain γ-lactones monitored via IR spectral analysis. Due to the formation of a solid mass, fraction 6 was exhaustively washed with cold ethanol until pure budlein A (500 mg) was obtained as white crystals. Its chemical structure was determined by means of spectrometric analysis, *i.e.* IR and nuclear magnetic resonance (NMR) spectrometry (^{1}H and ^{13}C), as well as comparison with authentic sample and data reported in the literature (Da Costa et al., 2001). The purity of budlein A was determined by chromatographic and spectrometric methods. TLC was carried out using several eluent systems and two spray reagents (1% vanillin–sulphuric acid or concentrated sulphuric acid). A high performance liquid chromatography (HPLC) run was made using methanol–water 55:45 or acetonitrile–water 65:35 as mobile phase, a reversed phase (ODS) analytical column, flow rate 1.0 or 1.3 mL/min, and UV detection at λ_{max} 225 and 265 nm, as described elsewhere (Da Costa et al., 2001). Only one compound was detected in the chromatographic analyses. The ^{13}C NMR spectrum of budlein A showed 20 carbon atoms corresponding to its structure. By means of chromatographic and spectrometric methods, we estimated that the purity of budlein A is between 95–98%, therefore suitable for these biological assays. HPLC chromatograms and NMR spectral data are available upon request.

2.2. Animals

Adult male Swiss mice (22–28 g) obtained from the University of Sao Paulo, campus of Ribeirao Preto, were housed in a temperature-controlled room, with access to water and food *ad libitum* until use. All experiments were double blind and conducted in accordance with the National Institute of Health guidelines on the welfare of experimental animals and with the approval of Ethics Committee of the Faculty of Medicine of Ribeirao Preto (University of Sao Paulo).

2.3. Paw oedema test

The volume of the mice paw was measured with a plesthismometer (Ugo Basil, Italy) before (V_o) the intraplantar stimulus

with carrageenan and 3 h after (V_T), as described previously (Winter et al., 1962). The amount of paw swelling was determined for each mouse and the difference between V_T and V_o was taken as the oedema value (oedema mm^3/paw). Paw oedema was also evaluated after complete Freund's adjuvant stimulus injection as described above.

2.4. Leukocyte migration tests

2.4.1. Leukocyte migration to the paw skin tissue

The myeloperoxidase (MPO) kinetic–colorimetric assay was used to evaluate the leukocyte migration to the subcutaneous plantar tissue of mice hind paw (Bradley et al., 1982; Casagrande et al., 2006). This method was also used to evaluate possible gastric damage (Souza et al., 2004). Samples of subcutaneous plantar tissue (Cunha et al., 2005) or stomach (Souza et al., 2004) were collected in 50 mM K_2HPO_4 buffer (pH 6.0) containing 0.5% hexadecyl trimethylammonium bromide (HTAB) and kept at −80 °C until use. Samples were homogenized using a Polytron (PT3100), centrifuged at 16,100 ×*g* for 4 min and the resulting supernatant assayed spectrophotometrically for MPO activity determination at 450 nm (Spectra max), with 3 readings in 1 min. The MPO activity of samples was compared to a standard curve of neutrophils. Briefly, 10 μl of sample were mixed with 200 μl of 50 mM phosphate buffer pH 6.0, containing 0.167 mg/ml *O*-dianisidine dihydrochloride and 0.0005% hydrogen peroxide. The results were presented as the MPO activity (number of neutrophils 10^4/paw).

2.4.2. Neutrophil migration to the peritoneal cavity

Neutrophil migration was assessed 4 h after carrageenan intraperitoneal stimulus. The animals were killed, and the cells present in the peritoneal cavity (cav) were harvested by introducing 3.0 ml of phosphate-buffered saline (PBS) containing 1 mM of EDTA. Total counts were performed with a cell counter (Coulter AC T series analyzer; Coulter Corp., Miami, USA), and differential cell counts were carried out on cytocentrifuge slides (Cytospin 3; Shandon Southern Products, Astmoore, UK) stained by the May–Grümwald–Giemsa (Rosenfeld) method. The results were expressed as the number of neutrophils/cavity (Secco et al., 2003).

2.5. Nociceptive tests

2.5.1. Writhing test

The antinociceptive activity was evaluated in mice using the writhing test (Collier et al., 1968). Acetic acid (0.6% v/v, 10 ml/kg) was injected into the peritoneal cavities of mice, which were placed in a large glass cylinder and the intensity of nociceptive behaviour was quantified by counting the total number of writhes occurring between 0 and 20 min after stimulus injection. The writhing response consists of a contraction of the abdominal muscle together with a stretching of the hind limbs. The antinociceptive activity was expressed as the writhing scores over 20 min.

2.5.2. Electronic pressure-meter test

We use the term hypernociception rather than hyperalgesia or allodynia to define the decrease in the nociceptive withdrawal threshold (Parada et al., 2003; Verri et al., 2006a). Mechanical hypernociception was tested in mice as previously reported (Cunha et al., 2004). In a quiet room, mice were placed in acrylic cages (12 × 10 × 17 cm) with wire grid floors, 15–30 min before the start of testing. The test consisted of evoking a hind paw flexion reflex with a hand-held force transducer (electronic anesthesiometer; IITC Life Science, Woodland Hills, CA) adapted with a 0.5 mm^2 polypropylene tip. The investigator was trained to apply the tip perpendicularly to the central area of the hind paw with a gradual increase in pressure. The end point was characterized by the removal of the paw followed by clear flinching movements. After the paw withdrawal, the intensity of the pressure was recorded automatically. The value for the response was an averaging of three measurements. The animals were tested before and after treatments. The results are expressed by delta (Δ) withdrawal threshold (in g) calculated by subtracting the zero-time mean measurements from the mean measurements 3 h after stimulus. Withdrawal threshold was 9.0 ± 0.5 g (mean ± SEM.; $n = 30$) before injection of the hypernociceptive agents (*e.g.* cytokines, carrageenan or complete Freund's adjuvant).

2.6. Measurement of motor performance

In order to discard possible non-specific muscle relaxant or sedative effects of budlein A, mice motor performance were evaluated on the rota-rod test (Rosland et al., 1990). The apparatus consisted of a bar with a diameter of 2.5 cm, subdivided into six compartments by disks 25 cm in diameter (Ugo Basile, Model 7600). The bar rotated at a constant speed of 22 rotations per minute. The animals were selected 24 h previously by eliminating those mice that did not remain on the bar for two consecutive periods of 180 s. Animals were treated with vehicle (tween 80 20% in saline) or budlein A (10 mg/kg, p.o) 30 min before testing, or were treated with this same dose for 8 days. The cut-off time used was 180 s.

2.7. Cytokine measurement

Three hours after the injection of carrageenan (100 μg/paw), animals were terminally anaesthetized, the skin tissues were removed from the injected and control paws (saline and naive). The samples were homogenized in 500 μl of the appropriate buffer containing protease inhibitors, and TNF-α, IL-1β and Keratinocyte-derived chemokine (KC) levels were determined as described previously (Cunha et al., 2005) by enzyme-linked immunosorbent assay (ELISA). The results are expressed as picograms (pg) of each cytokine per paw. As a control, the concentrations of these cytokines were determined in naive mice and animals injected with saline.

2.8. Experimental protocols

In all tests the animals were per orally (p.o.) treated with vehicle (tween 80 20% in water) or budlein A (1.0, 3.0 and 10.0 mg/kg) 30 min before stimuli. In only one series of experiments, mice received budlein A (10.0 mg/kg) at different

times (15, 6, 3 and 1/2 h) before the stimulus with carrageenan. Drug control groups were pre-treated with dexamethasone (1 h, 2.0 mg/kg, s.c.), indomethacin (40 min, 5 mg/kg, i.p.) or the respective vehicle (saline or tris (2-amino-2-hydroxymethyl-propan-1,3-diol)/HCl, pH 8.0, 200 μl). The inflammatory responses were evaluated as described above. The doses of inflammatory stimuli were previously determined in our laboratory in pilot studies based on previous works. The doses of carrageenan used were 50 μg/paw for oedema and leukocyte migration to the paw skin, 100 μg/paw for mechanical hypernociception and cytokines evaluation, 500 μg/cav for neutrophil migration to the peritoneal cavity. Other doses of mechanically hypernociceptive mediators used were TNF-α (100 pg/paw), KC (20 ng/paw), IL-1β (1 ng/paw), prostaglandin E_2 (PGE_2, 100 ng/paw) and dopamine (10 μg/paw) (Cunha et al., 2005). The dose of the acetic acid (0.6% v/v) used to induce abdominal contortions was 10 ml/kg (Collier et al., 1968). In another set of experiments, the effect of budlein A (10.0 mg/kg) on complete Freund's adjuvant (10 μl/paw)-induced hypernociception, paw oedema and paw skin MPO activity were evaluated.

2.9. Drugs and stimuli

The following materials were obtained from the sources indicated. The National Institute for Biological Standards and Control (NIBSC, South Mimms, Hertfordshire, UK) provided recombinant murine TNF-α and IL-1β. Recombinant murine KC was purchased from PeproTech INC., (Rocky Hill, NJ, USA), acetic acid from Mallinckrodt Baker, S.A. (Mexico, Mexico), carrageenan from FMC Corporation (Philadelphia, PA, USA), complete Freund's adjuvant and dexamethasone from Sigma (St. Louis, MO, USA), and indomethacin from Prodome (Campinas, SP, Brazil).

2.10. Statistical analyses

Results are presented as mean ± S.E.M. of experiments made on 5–7 animals per group. Differences between groups were evaluated by analyses of variance (one-way ANOVA) followed by Bonferroni's *t* test. Statistical differences were considered to be significant at $P<0.05$.

3. Results

3.1. Budlein A inhibits carrageenan-induced oedema and neutrophil migration to the paw skin and peritoneal cavity

The animals were per orally (p.o.) treated with budlein A (1.0, 3.0 or 10.0 mg/kg) and 30 min after carrageenan—(50 μg/paw) was injected intraplantarly (i.pl.). The paw oedema and leukocyte migration to the paw skin tissue (myeloperoxidase—MPO activity) were evaluated 3 h after stimulus (Fig. 2A and B), and Cg— (500 μg/cav) induced neutrophil migration to the peritoneal cavity 4 h after stimulus (Fig. 2c). Budlein A inhibited all the carrageenan-induced responses in a dose-dependent manner. The results obtained

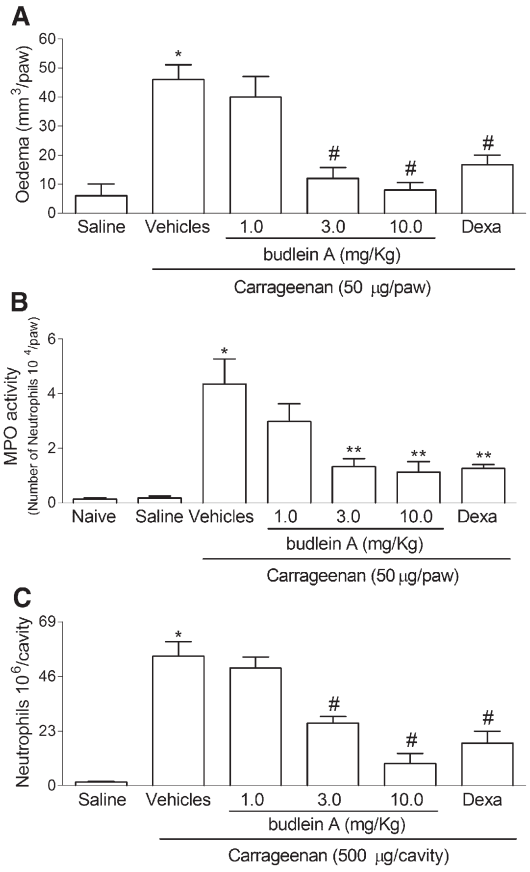

Fig. 2. Budlein A inhibits carrageenan-induced oedema and neutrophil migration to the paw skin and peritoneal cavity. Mice were treated with budlein A (1.0–10.0 mg/kg, p.o., 30 min, diluted in tween 80 20% in saline), dexamethasone (2 mg/kg, s.c., diluted in saline) or vehicles before: Panel A: Oedema was evaluated 3 h after stimulus with carrageenan (Cg, 50 μg/paw) intraplantar injection. Results are expressed as oedema in mm^3/paw ($n=5$). Panel B: Animals received the Cg (50 μg/paw) intraplantar stimulus, and the samples of subcutaneous plantar tissue were collected after 3 h. Results are expressed as myeloperoxidase activity (number of neutrophils 10^4/paw, $n=5$). Panel C: Peritoneal exudates were collected 4 h after carrageenan (Cg, 500 μg/cavity) intraperitoneal stimulus. Total counts were performed with a cell counter and differential cell counts were carried out on cytocentrifuge slides stained by Rosenfeld method. Results are expressed as number of neutrophil 10^6/cavity ($n=6$). * $P<0.05$ compared with the saline group, ** $P<0.05$ compared to the vehicle group, and # $P<0.05$ compared to the vehicle group and the dose of 1.0 mg/kg group (One-way ANOVA followed by Bonferroni's *t* test).

with the control groups support the effects of budlein A since the vehicle (tween 80 20% in saline) presented no activity, and the control drug dexamethasone (2 mg/kg, s.c., 1 h) inhibited the carrageenan-induced paw oedema, and the neutrophil migration to the paw skin and peritoneal cavity (Fig. 2).

3.2. Budlein A inhibits nociception while not affecting the direct PGE_2- or dopamine-induced hypernociception

Budlein A dose— (1.0, 3.0 or 10.0 mg/kg) dependently inhibited the acetic acid-induced writhes as presented by the cumulative number of writhes over 20 min (Fig. 3A). The results obtained with the control groups confirm the effects of

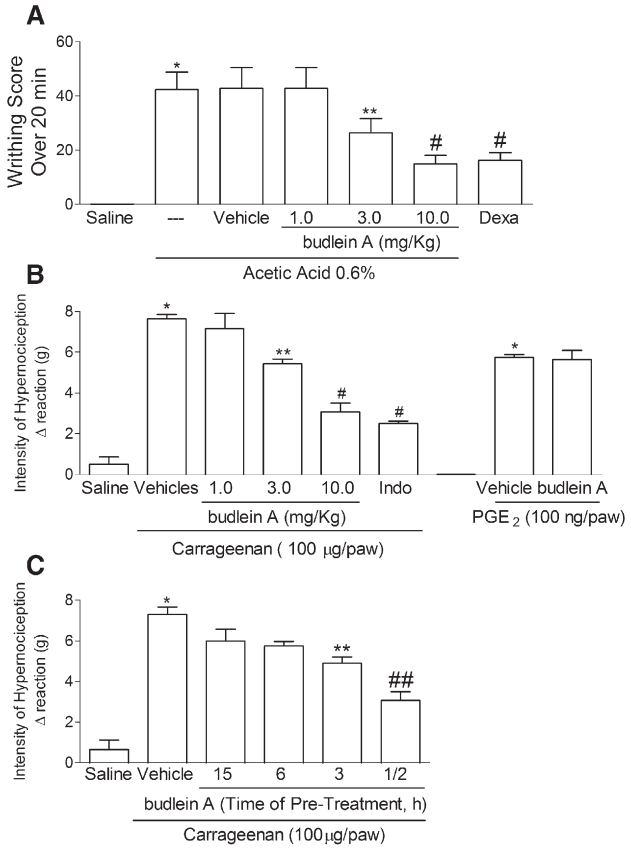

Fig. 3. Budlein A inhibits nociception while not affects the direct PGE_2-induced hypernociception. Panel A: Mice were treated with budlein A (1.0–10.0 mg/kg, p.o., 30 min, diluted in tween 80 20% in saline), dexamethasone (2 mg/kg, s.c., diluted in saline) or vehicles before the i.p. injection of acetic acid (AcAc, 0.6%). The writhings were evaluated over 20 min ($n=7$). Panel B: Left bars, mice were treated with budlein A (same dose), indomethacin (5 mg/kg, s.c., diluted in Tris/HCl, pH 8.0) or vehicles before the Cg (100 μg/paw) injection. Right bars, mice were treated with budlein A (same dose) or vehicle before the PGE_2 (100 ng/paw) injection. Panel C: Mice were treated with budlein A (10.0 mg/kg, p.o., 0.5–15 h) or vehicle before the carrageenan (100 μg/paw) injection. For panels B and C the intensity of hypernociception was measured 3 h after stimulus injection by the electronic pressure-meter test ($n=4$–5). * $P<0.05$ compared with the saline group, ** $P<0.05$ compared to the vehicle group, and # $P<0.05$ compared to the vehicle group and the dose of 1.0 mg/kg group (panels A and B) ## $P<0.05$ compared to the vehicle gro and the 15 h of pre-treatment group (panel C) (One-way ANOVA followed by Bonferroni's t test).

budlein A since the vehicle (tween 80 20% in saline) presented no activity, and the control drug dexamethasone (2 mg/kg, s.c., 1 h) also inhibited the acetic acid-induced writhes. The antinociceptive activity of budlein A was further investigated using the electronic-pressure meter (Cunha et al., 2004). Budlein A pre-treatment dose-dependently (1.0, 3.0 or 10.0 mg/kg, 30 min) inhibited the carrageenan (100 μg/paw) induced mechanical hypernociception in the third hour (Fig. 3B), which was not affected by vehicle treatment (tween 80 20% in saline). Moreover, the pre-treatment with budlein A at different times (15, 6, 3 and 1/2 h) demonstrated that it is effective even when administrated 3 h before the Cg injection, and evaluation 3 h after stimulus (at least 6 h elapsed period) (Fig. 3C). The control drugs dexamethasone (2 mg/kg/s.c., 1 h before) and indomethacin (5 mg/kg/i.p., 40 min before) also inhibited carrageenan hypernociception and acetic acid-induced writhes, respectively (Fig. 3, panels A and B). On the other hand, budlein A (10.0 mg/kg, 30 min) did not inhibit PGE_2— (100 ng/paw, Fig. 3B, right bars) or dopamine— (10 μg/paw, data not shown) induced hypernociception. These results suggest that budlein A does not act inhibiting the hypernociception induced by final hypernociceptive mediators (PGE_2 or dopamine) that act directly on their receptors on nociceptive fibers. Thus, budlein A mechanism of action might be on the production of inflammatory hypernociceptive mediators.

3.3. Budlein A inhibits carrageenan-induced cytokine production and cytokine-induced mechanical hypernociception

Budlein A pre-treatment (10.0 mg/kg, 30 min) inhibited the carrageenan— (100 μg/paw) induced release of TNF-α, KC and IL-1β in the mice paw tissue 3 h after stimulus (Fig. 4A, B and c, respectively). Further supporting the anti-inflammatory

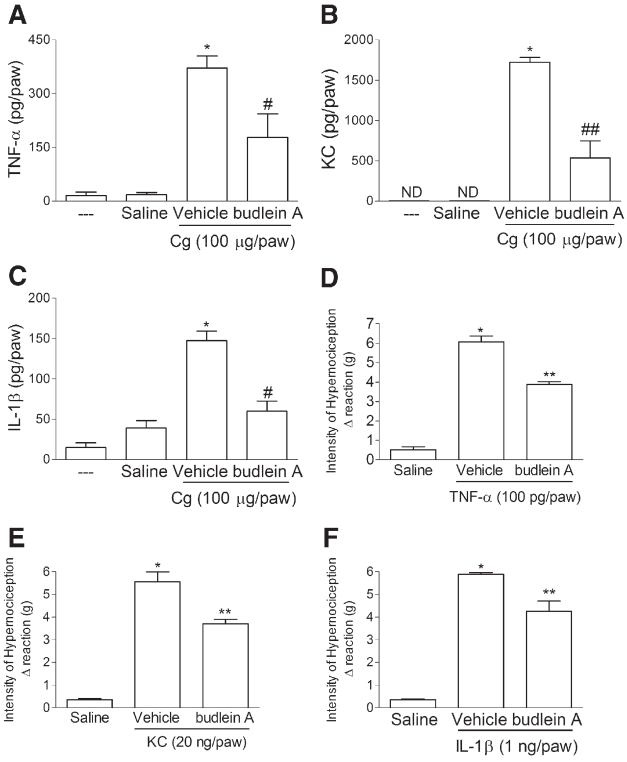

Fig. 4. Budlein A inhibits carrageenan-induced cytokine production and cytokine-induced mechanical hypernociception. Panels A–C: Mice were treated with budlein A (10.0 mg/kg, p.o., 30 min) or vehicle (tween 80 20% in saline) before the carrageenan (Cg, 100 μg/paw) stimulus. The samples of subcutaneous plantar tissue were collected 3 h after stimulus and processed for TNF-α (Panel A), KC (Panel b) and IL-1β (Panel C) levels measurement ($n=3$–4). Panel D–F: Mice were treated with budlein A (10.0 mg/kg, p.o., 30 min) or vehicle (tween 80 20% in saline) before the hypernociceptive stimulus with TNF-α (Panel D), KC (Panel E) or IL-1β (Panel F). The intensity of hypernociception was measured 3 h later by the electronic pressure–meter test ($n=5$). * $P<0.05$ compared to the saline group (All panels), # $P<0.05$ compared to the vehicle group, and not statistically different from saline group (panels A and C), ## $P<0.05$ compared to the vehicle and saline groups (panels D–F), and ** $P<0.05$ compared to the vehicle group (panels D–F) (One-way ANOVA followeed by Bonferroni's t test). Not detectable, ND (panel B).

activity of budlein A, using this same treatment schedule, it inhibited the TNF-α- (100 ng/paw) and KC- (20 ng/paw), and IL-1β- (1 ng/paw) induced mechanical hypernociception in the third hour (Fig. 4D, E and F, respectively).

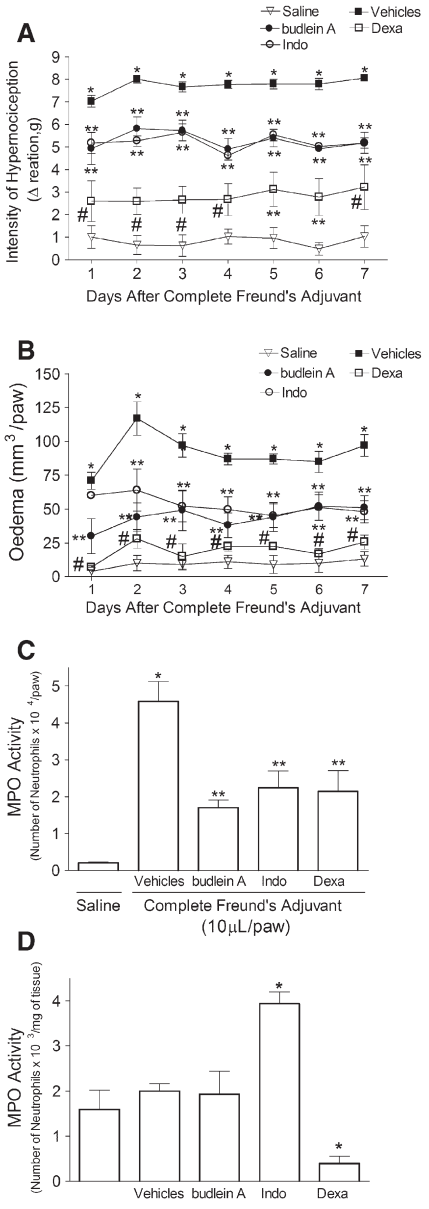

Fig. 5. Budlein A treatment inhibits Complete Freund's Adjuvant-induced inflammation. Panels A–D: Mice were treated with budlein A (10.0 mg/kg, p.o., diluted in tween 80 20% in saline.), indomethacin (5 mg/kg, p.o., diluted in Tris/HCl, pH 8.0), dexamethasone (2 mg/kg, s.c., diluted in saline) or vehicles during 7–8 days. The treatment started 30 min before Complete Freund's Adjuvant (CFA, 10 μL/paw) stimulus. Thereafter, mice ($n=5$) were treated with one of the above mentioned drugs once a day, 3 h before measurements. Saline group received 10 μL/paw of saline instead of Complete Freund's Adjuvant. The mechanical hypernociception (Electronic pressure meter, Panel A) and oedema (Panel B) were evaluated until the 7th day. In the eighth day, samples of the subcutaneous plantar tissue (Panel C) and stomach (Panel D were collected 3 h after the last treatment for MPO activity evaluation. * $P<0.05$ compared to the saline group, ** $P<0.05$ compared to the vehicles group, and # $P<0.05$ compared to the vehicles group and not statistically different of the saline group (One-way ANOVA followed by Bonferroni's t test).

3.4. Budlein A treatment inhibits complete Freund's adjuvant-induced inflammation

In general, anti-inflammatory drugs are used chronically or during short periods such as 7 days. However, even during those short periods conventional non-steroidal anti-inflammatory drugs (cyclooxigenase-1 inhibitors) may cause gastric damage, which restrict their use (Gudis and Sakamoto, 2005). Therefore, the budlein A anti-inflammatory activity was tested in the complete Freund's adjuvant (10 μl/paw)-induced inflammation since the inflammation in this model persists for more than 8 days. This period of treatment allows evaluating the effect on stomach inflammation. Mice were treated once a day (10 mg/kg) during 8 days with budlein A (10 mg/kg), indomethacin (indo, 5 mg/kg/p.o.) or dexamethasone (2 mg/kg/s.c.). Mechanical hypernociception (Fig. 5A) and oedema (Fig. 5B) were evaluated between the first and seventh days. In the eighth day, the motor performance of the mice was evaluated in the rotarod. After, those mice were sacrificed and samples of the stomach (Fig. 5C) and paw skin (Fig. 5D) were colleted for MPO activity assay. Budlein A inhibited the complete Freund's adjuvant-induced mechanical hypernociception, paw oedema and increase of MPO activity in the paw skin. The budlein A treatment did not alter the MPO activity in the stomach sample, which is consistent with no inflammatory response in the tissue (Souza et al., 2004). The motor performance was not altered either few hours (30, 90 and 210 min) after single treatment or after 8 consecutive days of budlein A administration (data not shown). Dexamethasone treatment reduced all inflammatory responses. Indomethacin treatment reduced the mechanical hypernociception, paw oedema and paw MPO activity. However, consistent with the inhibition of cyclooxigenase-1 and inflammation, the indomethacin treatment increased the MPO activity in the stomach. None of the treatments induced macroscopic gastric lesions (data not shown), which would be especially intriguing concerning indomethacin treatment. Nevertheless, there is evidence that the per oral treatment with indomethacin or other cycloxigenase inhibitors (*e.g.* acetyl salicylic acid) induce gastric mucosa tolerance described as no detectable gastric mucosal lesions concomitantly with an increase in polymorphonuclear cell infiltrate as determined by the MPO activity (Wallace et al., 1995).

4. Discussion

V. robusta is a perennial herb found widespread in Brazil. The Mexican folk medicine uses other plants of this species and related genera (Heinrich et al., 1998). In the present study, we isolated the sesquiterpene lactone budlein A from the dichloromethane extract of *V. robusta*, and demonstrated its *in vivo* anti-inflammatory activity, suggesting the inhibition of cytokines release and action as its mechanism.

Budlein A dose-dependently inhibited the carrageenan-induced oedema, neutrophil migration to the paw skin and peritoneal cavity, and acetic acid-induced writhes. These were seminal methods for the *in vivo* evaluation of the activity of drugs such as steroidal and non-steroidal anti-inflammatory,

inhibitors of leukotriene synthesis and cytokine directed antibodies (French and Galicich, 1964; Limet and Lecomte, 1968; Goldman et al., 1993; Canetti et al., 2001).

Focusing in nociception, using the electronic pressure-meter test (Cunha et al., 2004; Vivancos et al., 2004) we have recently demonstrated that carrageenan initiates a mechanical hypernociceptive cascade in which prostanoids and sympathetic amines are the final mediators that induce nociceptor sensitization in mice. Moreover, cytokines mediate the release of those final mediators induced by carrageenan. Thus, after carrageenan stimulus TNF-α and KC have pivotal roles in a cytokines cascade: i.) TNF-α → IL-1β that activates the synthesis of prostanoids, ii.) KC → sympathetic amines release, and iii.) KC → IL-1β → prostanoids production (Cunha et al., 2005).

Budlein A dose-dependently inhibited carrageenan-induced mechanical hypernociception. However, it did not affect the hypernociception induced by the final mediators PGE_2 and dopamine. In addition, budlein A treatment inhibited the carrageenan-induced release of TNF-α, KC and IL-1β as well as the hypernociception induced by these cytokines. The inhibition of cytokines release might ultimately lead to the inhibition of prostanoids and sympathetic amines production/release. Substantiating this hypothesis, the treatment with indomethacin (standard cyclooxigenase inhibitor) inhibits the hypernociceptive effect of TNF-α, KC and IL-1β, and guanethidine (sympathetic blocker) inhibits KC hypernociception in mice (Cunha et al., 2005). Additionally, considering the inhibition of NFκB activation by sesquiterpene lactones (Hehner et al., 1998; Siedle et al., 2004) it is also possible that budlein A inhibits inflammation-induced cyclooxigenase-2 mRNA expression, and therefore, PGE_2 production. This mechanism may account for the inhibition of IL-1β-induced mechanical hypernociception since it depends on PGE_2 production. Thus, the results presented above suggest that budlein A acts preventing the nociceptor sensitisation by inhibiting the production of mediators that sensitise the nociceptor. Furthermore, these same mediators (*e.g.* TNF-α, IL-1β, KC, prostanoids, sympathetic amines) are also involved in the acetic-acid-induced writhes (Ribeiro et al., 2000; Cunha TM, unpublished data). Therefore, the inhibition of cytokine production might also account for the antinociceptive effect of budlein A in the writhing test.

Further supporting the applicability of budlein A as an anti-inflammatory drug, it inhibited the complete Freund's adjuvant-induced hypernociception, paw oedema and increase of paw skin MPO activity during a 7–8 days treatment schedule. These effects were not accompanied by altered liver appearance or weight (data not shown), or gastric damage as determined by MPO activity. Furthermore, disproving relaxing or anaesthetic effects, budlein A (10.0 mg/kg) treatment did not affect the motor performance of the animals as tested in the rota-rod neither acutely (one treatment) or chronically (8 days treatment) (data not shown). Reinforcing this issue, the treatment with budlein A did not affect the mechanical hypernociceptive responses to PGE_2 and dopamine. Thus, budlein A may be useful at least for short-term therapies without the side effects of cyclooxigenase-1 inhibitors such as gastric damage.

The molecular mechanisms of action of sesquiterpene lactones other than budlein A, involve the alkylation of the p65 subunit cystein residue of the NF–kB complex, which inhibits its interaction with DNA, and thus, disabling its transcription (Rüngeler et al., 1999). Additionally, other sesquiterpene lactones also specifically inhibit the activation of NF–kB by preventing the degradation of I–kB. Moreover, other sesquiterpene lactones (santonin, isophoronoxide and sclareolide) prevent the activation of NF–kB and the degradation of I–kB induced by different stimuli such as phorbol esters, TNF-α and hydrogen peroxide in cultured Jurkat leukemia (Hehner et al., 1998). The most clinically used inhibitors of NF–kB are the glucocorticosteroids such as dexamethasone (control drug used in the present study). The inhibition of NF–kB or stimulation of I–kB production, and the interaction of glucocorticoids with the glucocorticoid responsive genes ultimately lead to the inhibition of cytokine production and action as well as genes encoding pro-inflammatory molecules and enzymes (Ferreira et al., 1997; Baeuerle and Henkel, 1994; Baeuerle and Baltimore, 1996; May and Ghosh, 1998, for review see Barnes, 2006). This is an important anti-inflammatory mechanism considering that cytokines induce hypernociception (Ferreira et al., 1988; Cunha et al., 1991, 1992; Verri et al., 2004, 2005, 2006b, in press) and neutrophil migration (Canetti et al., 2001; Sayers et al., 1988). In agreement with our results, budlein A inhibits NF–kB activation as determined using the electrophoretic mobility shift assay (Siedle et al., 2004). It is important to point out that glucocorticosterois bind to glucocorticoid cytoplasmatic receptors, and then the complex induce the known effects and side effects of steroids. Therefore, budlein A probably does not present the common side effects associated with glucocorticosteroids therapy since sesquiterpene lactones bind on NF–kB or I–kB.

Concluding, the present study demonstrated that budlein A inhibits the inflammation signs by a mechanism related to the inhibition of cytokines release and action. Thus, budlein A might be a useful orally active drug to control inflammatory conditions involving hypernociception, oedema and neutrophil migration.

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