SUPPLEMENTARY MATERIAL

Chenopodin as an anti-inflammatory compound

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Abstract: Chenopodin is an 11S-type globulin purified from *Chenopodium quinoa* seeds, which can bind carbohydrates and hemagglutinating human erythrocytes. The present study aimed to evaluate the N-terminal structure of the heterodimeric Chenopodin and its effects in models of inflammation. Chenopodin presented two subunits on its structure and has N-terminal homology with other Chenopodin in 92%. Chenopodin decreased paw edema and neutrophil recruitment induced by carrageenan in mice. Concluding, we demonstrated that Chenopodin exhibits *in vivo* anti-inflammatory activity.

Keywords: Chenopodin; *Chenopodium quinoa*; seeds; anti-inflammatory activity; *in vivo*

Experimental

Materials

The materials used in this study were: *Chenopodium quinoa* seeds genotype BRS Syetetuba (EMBRAPA, Brazil, Lot. B2012); λ-carrageenan (Sigma-Aldrich, St. Louis, MO, USA; Lot # 0001408463); Phenylmethylsulfonyl fluoride (Sigma-Aldrich, St. Louis, MO, USA; Lot. BCCB9214); Benzethonium chloride (Sigma-Aldrich, St. Louis, MO, USA; Lot. SLBS1093V); EDTA (Sigma-Aldrich, St. Louis, MO, USA; Lot. BCCD7594); Aprotinin A (Sigma-Aldrich, St. Louis, MO, USA; Lot. SLBX1798); Tween-20 (Sigma-Aldrich, St. Louis, MO, USA; Lot. SLCC6910); TNF-alpha kit (R&D Systems, Minneapolis, MN, USA; Lot. P116661); May-Grunwald solution (Renylab, Barbacena, MG, Brazil; Lot. 20050556); Giemsa solution (enylab, Barbacena, MG, Brazil; Lot. 20050539); NaCl (Synth, Diadema, SP, Brazil; Lot. 228582); Na2HPO4 (Synth, Diadema, SP, Brazil; Lot. 239850); Sodium dodecyl sulfate (Amresco, Solon, Ohio, EUA; Lot. M107); and PVDF membranes (GE Healthcare, Little Chalfont, UK, Lot. RPN303F).

Protein purification and N-terminal analysis

Chenopodin was obtained from *C. quinoa* seeds using standard protein purification techniques as described previously (Pompeu *et al*., 2015). Reduced forms of Chenopodin (50 µg) from sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) were electroblotted onto PVDF membranes and the desired bands were excised and sequenced on an Applied Biosystems model Precise 491 protein sequencer coupled to a 120-A PTH amino acid analyzer (Foster City, CA, USA). Basic Local Alignment Search Tool (BLAST) was used to align the homologous sequences to the protein of interest and compare to other N-terminal sequences.

Anti-inflammatory assays of Chenopodin

Animals

Male Swiss mice (7-8 weeks old, 25-30 g) with free access to food and water were used. The animals were kept in a room with a 12 h light-dark cycle for at least 3 days before the experiment to allow for acclimatization. A room temperature of 27°C was used, which corresponds to the thermoneutral zone for mice. This study was approved by the Ethics Committee on Animal Experimentation of the Federal University of Minas Gerais (Protocol 237/2016) and all experiments were conducted according to the ethical guidelines for investigation of experimental pain in conscious animals.

Drugs

Suspensions of carrageenan were prepared in sterile saline (used for intraplantar injection, 30 μL volume) or in PBS (used for intrapleural injection, 100 μL volume). The volume of intravenous (i.v.) administration of sterile saline or Chenopodin was 100 μL. All solutions and suspensions were prepared immediately before use.

Paw edema induced by carrageenan

Paw volume was measured with a plethysmometer (Model 7140, Ugo Basile S.R.L., Italy). The basal volume of the right hind paw was measured before any treatment. Next, the animals were divided into the experimental groups in such a way that the mean volumes of the different groups were similar. Carrageenan (600 μg, 30 μL) or vehicle (sterile saline, 30 μL) was injected via the intraplantar route. Vehicle (sterile saline, 100 μL) or Chenopodin (0.1, 1.0, and 10.0 mg/kg) was administered i.v. 30 min before the injection of carrageenan. The paw volume of each animal was again measured at 2, 4 and 6 h after injection of the inflammatory stimulus. The results were expressed as the paw volume changes (μL) in relation to the basal values.

Leukocyte recruitment to the pleural cavity induced by carrageenan

Leukocyte recruitment to the pleural cavity was evaluated according to Klein (Klein *et al*., 2001) and Matsui (Matsui *et al*., 2015). Mice were treated with Chenopodin (i.v.; 0.1, 1.0, and 10.0 mg/kg) or vehicle (sterile saline, 100 µL), 30 min before injection of the inflammatory stimulus. Carrageenan (200 µg, 100 µL) or vehicle (PBS; 100 µL) was injected via the intrapleural route and the animals were euthanized after 4 h. The cells present in the cavity were harvested by injecting 2 mL of PBS followed by collection of the exudate. Total cell counts were performed in a modified Neubauer chamber using Turk's stain. Differential cell counts were performed on cytospin preparations, which were stained with May-Grunwald and Giemsa to identify cell types according to standard morphological criteria.

TNF-α concentrations

The concentration of TNF-α was measured in the paw tissue of animals using commercially available antibodies, according to the procedures supplied by the manufacturer (R&D Systems, Minneapolis, MN, USA). Vehicle or Chenopodin (10.0 mg/kg) were administered i.v. 30 min before the injection of carrageenan (600 µg, 30 µL). Four hours after carrageenan injection, the animals were euthanized and the soft paw tissue was collected. These samples were weighed and homogenized in 1 mL of PBS (0.4 M NaCl and 10 mM $NaPO_4$) containing proteases inhibitors (0.1 mM phenylmethylsulfonyl fluoride, 0.1 mM benzethonium chloride, 10 mM EDTA, and 2 µg/mL aprotinin A) and 0.05% Tween-20 and centrifuged (3,000 rpm) for 10 min at 4 °C. The supernatant was stored at – 70 °C until analysis of TNF-α concentration. The supernatant samples were used for an enzyme-linked immunosorbent assay at a 1:3 dilution in PBS. All samples were assayed in duplicate and the results were expressed as pg/100 mg of tissue.

Statistical analysis

Results are presented as mean ± standard error of the mean. Differences were evaluated using one- or two-way analysis of variance followed by Newman–Keuls or Bonferroni *post-hoc* tests. A *p*-value < 0.05 was considered significant and statistical analysis was conducted using GraphPrism 5.0 for Windows.

Figures

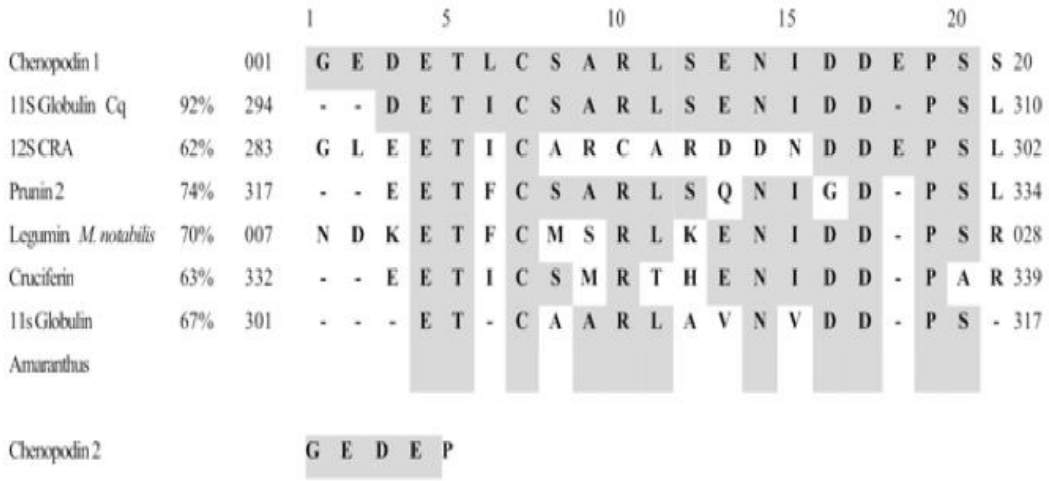

Figure S1. N-terminal analysis of Chenopodin. Chenopodin purified from the *C. quinoa* variety BRS Syetetuba has 92% homology with the previously published N-terminal sequence (unknown variety).

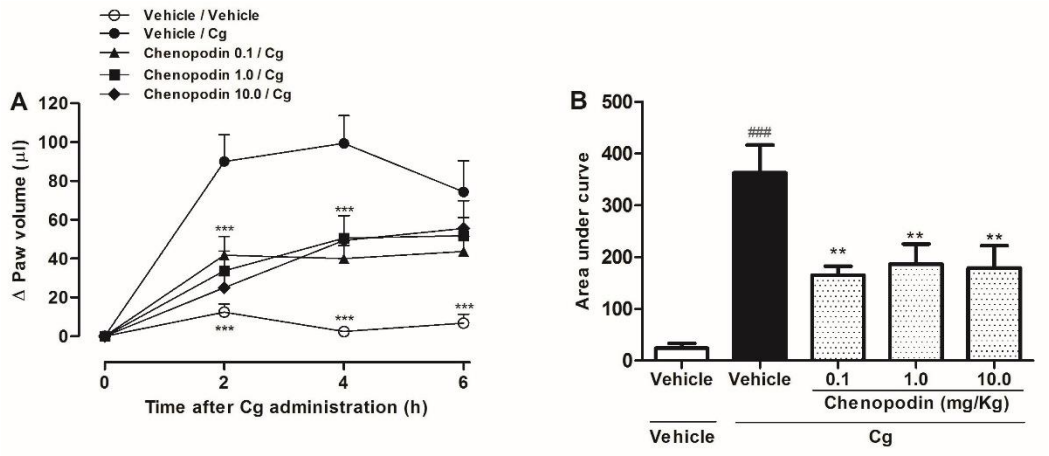

Figure S2. Effects induced by Chenopodin (0.1, 1.0, or 10.0 mg/kg, i.v., -30 min) or vehicle (sterile saline, 100 µl, i.v., -30 min) on the edema induced by intraplantar injection of carrageenan (Cg, 600 µg/paw). The negative control group received i.v. and intraplantar injection of sterile saline. Each point represents the mean ± standard error of the mean (SEM) of 8 animals. A represents the temporal course and B represents the area under the curve. ** and *** indicate a significant difference compared with Cg-treated group ($p<0.01$ and $p<0.001$, respectively). ### significantly different compared to the vehicle alone group ($p<0.001$).

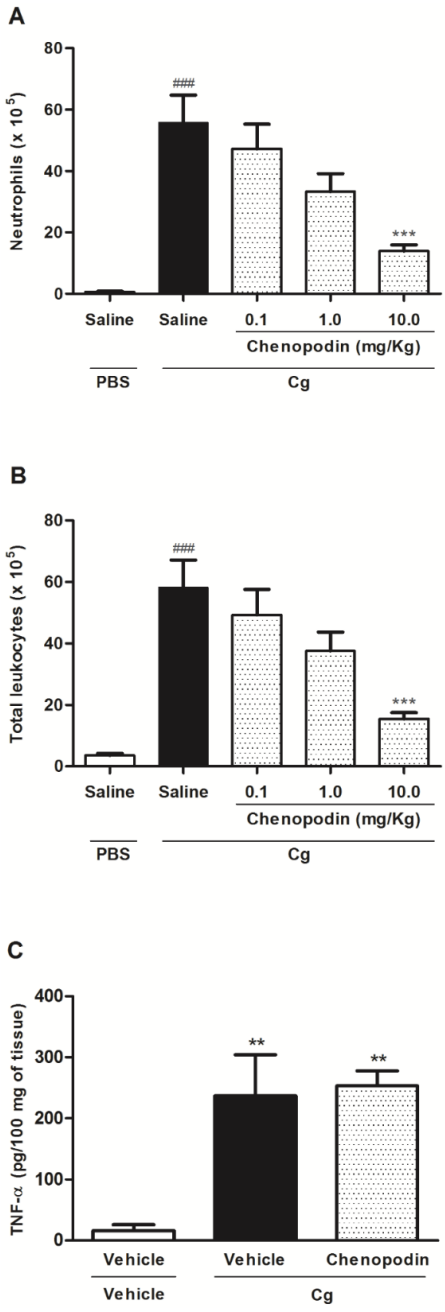

Figure S3. Effect induced by Chenopodin (0.1, 1.0, or 10.0 mg/kg, i.v., -30 min) on neutrophil (A) and total leukocyte (B) recruitment induced by intrapleural injection of carrageenan (Cg, 200 μg). Cell recruitment was evaluated 4 h following injection of Cg. Each point represents the mean ± SEM of 5–6 animals. ### indicates a significant difference compared with the saline/PBS-treated group ($p<0.001$). *** indicates a significant difference compared with the saline/Cg-treated group ($p<0.001$). (C) Effect induced by Chenopodin (0.1, 1.0, or 10.0 mg/kg, i.v., -30 min) on paw tissue production of TNF-α induced by intraplantar injection of carrageenan (Cg, 600 μg/paw). The concentration of TNF-α was evaluated 4 h following injection of the inflammatory stimulus. Each bar represents the mean ± SEM of 5–6 animals. ** significantly different compared to the vehicle alone group ($p<0.01$).

References

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