Original Research

Crotoxin, a rattlesnake toxin, induces a long-lasting inhibitory effect on phagocytosis by neutrophils

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Abstract

Crotalus durissus terrificus snake venom (CdtV) has long-lasting anti-inflammatory properties and inhibits the spreading and phagocytic activity of macrophages. Crotoxin (CTX), the main component of CdtV, is responsible for these effects. Considering the role of neutrophils in the inflammatory response and the lack of information about the effect of CdtV on neutrophils, the aim of this study was to investigate the effect of CdtV and CTX on two functions of neutrophils, namely phagocytosis and production of reactive oxygen species, and on the intracellular signaling involved in phagocytosis, particularly on tyrosine phosphorylation and rearrangements of the actin cytoskeleton. Our results showed that the incubation of neutrophils with CdtV or CTX, at different concentrations, or the subcutaneous injection of CdtV or CTX in rats two hours or one, four or 14 days before or one hour after the induction of inflammation inhibited the phagocytic activity of neutrophils. Furthermore, these *in vitro* and *in vivo* effects were associated with CdtV and CTX inhibition of tyrosine phosphorylation and consequently actin polymerization. Despite the inhibitory effect on phagocytosis, this study demonstrated that CdtV and CTX did not alter the production of the main reactive oxygen species. Therefore, this study characterized, for the first time, the actions of CdtV on neutrophils and demonstrated that CTX induces a long-lasting inhibition of tyrosine phosphorylation and consequently phagocytosis. We suggest that CTX represents a potential natural product in controlling inflammatory diseases, since a single dose exerts a long-lasting effect on intracellular signaling involved in phagocytosis by neutrophils.

Keywords: *Crotalus durissus terrificus* venom, crotoxin, neutrophil, phagocytosis, tyrosine phosphorylation, actin polymerization

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Introduction

The venom of the South American rattlesnake *Crotalus durissus terrificus* (CdtV) is a complex mixture of biologically active substances such as toxins (crotamine, crotoxin [CTX], gyroxin and convulxin), enzymes and peptides.[1] CTX is the major component of the venom; this toxin of well-known structure is a complex of two proteins, namely a basic phospholipase A_2 with weak toxicity (component B; CB) and an acidic, enzymatically inactive, non-toxic protein known as crotapotin (component A; CA), which potentiates the toxicity of phospholipase A_2 by acting as a chaperone protein.[2,3]

Several studies have demonstrated that CdtV and CTX modulate different aspects of the immune and inflammatory response.[4] Mice pretreated with CTX and immunized with human serum albumin or ovalbumin produced lower levels of IgG1 antibodies than did control mice. This down-modulating effect was also observed in proliferation of T lymphocytes and cytokine secretion *in vitro*.[5–7] Furthermore, our previous studies demonstrated that CTX has long-lasting anti-inflammatory effects; a single injection of this toxin inhibits paw edema, cell migration and leukocyte-endothelium interactions induced by carrageenan (Cg). This anti-inflammatory effect is mediated by the activation of formyl-peptide receptors, decrease in the expression of adhesion molecules (P-selectin and ICAM-1) and inhibition of the secretion of proinflammatory cytokines.[8–11]

It has also been demonstrated that CdtV and CTX modulate macrophage function, decreasing cell spreading and phagocytosis and increasing candidacidal activity, hydrogen peroxide and nitric oxide production, and glucose and glutamine metabolism.[12–14] The inhibitory effect on

phagocytosis by macrophages involves increased F-actin re-organization and also inhibition of Rac and RhoA GTPase activity.[15] Despite these lines of evidence, the effect of CdtV on neutrophil functions has not yet been investigated. Models of Cg-induced inflammatory response have been widely used to investigate the pathophysiology of acute inflammation and to evaluate functions of neutrophils.[16,17]

Neutrophils play a key role in the mechanism of host defense, and their main functions in inflammation include the phagocytosis and degradation of the harmful agent. Neutrophils, along with macrophages, comprise the professional phagocytes; at the inflammatory site, neutrophils and macrophages, recruited by the action of chemotactic agents, initiate the process of phagocytosis of the harmful agent and cellular debris; neutrophils are the first cells that migrate to the site of injury.[18,19]

The phagocytic process comprises several sequential and complex events initiated by the recognition of ligands on the surface of particles by specific receptors, such as FcγR and CR3 on the surface of phagocytic cells. The clustering of these receptors, after binding to particles, is followed by the activation of signaling pathways, where actin microfilaments are polymerized, filling pseudopods that first engulf bound particles and later fuse into nascent phagosomes. Various molecular mechanisms are involved in this process; during CR3-mediated phagocytosis, small GTPases of the Rho family, particularly RhoA, are key factors in the regulation of actin dynamics. In addition to GTPases, tyrosine phosphorylation of multiple proteins by intracellular tyrosine kinases is an early and important signaling event of phagocytosis.[20]

Because of the importance of neutrophils in the inflammatory response, the modulating effect of CdtV on this response, and the lack of information regarding the effect of CdtV on neutrophils, the aim of this study was to investigate the effect of CdtV and CTX on two neutrophil functions, namely phagocytosis and production of reactive oxygen species, and on the intracellular signaling involved in phagocytosis, particularly the tyrosine phosphorylation of signaling proteins and re-arrangements of the actin cytoskeleton.

Material and methods

Animals

Male Wistar rats, weighing between 160 and 180 g, were used in this study. All procedures were in accordance with the guidelines for animal experimentation and were approved by the Institutional Animal Care Committee of the Butantan Institute (CEUAIB, protocol numbers 321/2006, 407/2007 and 705/2010).

Venom

A pool of lyophilized venom was obtained at the Laboratory of Herpetology of Butantan Institute from various adult specimens of *C. durissus terrificus* snakes of different gender and age from São Paulo, southeast region of Brazil. Venom was manually extracted by squeezing the glands, collected in Petri dishes, frozen and lyophilized.[21] The lyophilized venom pool was stored at −20°C and the venom solutions were prepared (w/v) in sterile saline (0.85% NaCl) or RPMI 1640 medium at the moment of use.

CTX

CTX was provided by Dr Maisa S Della Casa. Crude venom solution was submitted to anion-exchange chromatography as previously described by Rangel-Santos *et al.*,[22] using a Mono-Q HR 5/5 column in a FPLC system (Pharmacia, Uppsala, Sweden). Fractions (1 mL/min) were eluted using a linear gradient of NaCl (0–1 mol/L in 50 mmol/L Tris-HCl, pH 7.0). Three peaks (p1, p2 and p3) were obtained, and p2 corresponded to the pure CTX fraction (about 60% of the crude venom); peaks 1 and 3 included the other CdtV toxins. Prior to pooling, fractions containing CTX were checked for homogeneity by non-reducing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (12.5%),[23] and phospholipase A_2 activity was assessed by a colorimetric assay using a synthetic chromogenic substrate.[24]

Peritoneal exudate

Peritoneal exudate was obtained by an intraperitoneal injection of Cg (Sigma, St Louis, MO, USA) (4.5 mg/kg) before or after treatment with CdtV or CTX. Animals were sacrificed four hours after Cg injection in a CO_2 chamber, and the peritoneal cavity was washed with 10 mL of sterile cold phosphate-buffered saline (PBS), pH 7.4. After gentle massage of the abdominal wall, the peritoneal fluid containing neutrophils was collected in a plastic syringe. Total peritoneal cell count was determined with a Neubauer hemocytometer, and differential counts were performed after panchromatic staining,[25] showing that 95% of the cells were neutrophils.

Phagocytic activity of neutrophils

Neutrophils (1×10^6/mL) were incubated with 1 mL of RPMI 1640 medium containing opsonized zymosan particles for 40 min, at 37°C, in an atmosphere containing 5% CO_2. Phagocytic activity was determined in smears stained with a panchromatic dye.[25] A total of 100 cells were counted by light microscopy. Different scores were given to the number of neutrophils that had phagocytized no zymosan particles (×0), one particle (×1), two particles (×2) or three or more particles (×3). The index of phagocytic activity was calculated by the sum of the scores obtained per rat.[26]

Opsonization of zymosan particles (Saccharomyces cerevisiae)

Zymosan particles (Sigma), obtained from yeast cell walls, were suspended in PBS providing a concentration of particles of 2.8 mg/mL. For opsonization, zymosan particles were mixed with normal rat serum and incubated for 30 min at 37°C. The opsonized zymosan particles were

then centrifuged and suspended in RPMI 1640 medium for the phagocytosis assay. The number of opsonized zymosan particles was approximately 5×10^6 for each assay (five particles per cell).

Immunocytochemical assays

Neutrophils (1×10^6/mL) were incubated with opsonized zymosan particles for five or 15 min, at 37°C, in an atmosphere containing 5% CO_2. At the end of the assay, the cells were fixed in 4% paraformaldehyde–0.1 mol/L phosphate buffer (PB) and permeabilized with 0.2% Triton X-100 for 10 min at room temperature. After rinsing with PB, the cells were postfixed in 95% ethanol for five minutes at −20°C. Neutrophils were rinsed with PB and re-hydrated with PB containing 0.5% non-immune goat serum (Sigma) for 30 min at room temperature, to block non-specific binding sites. Incubation with the primary antibody against phosphotyrosine (anti-PTry; Sigma) diluted 1:100 in PB was performed overnight at room temperature. After rinsing, neutrophils were incubated with fluorescein isothiocyanate-labeled secondary antibody (goat antimouse IgG; Sigma) diluted 1:200 in PB, for 90 min at room temperature. The cells were then rinsed and stained for 30 min with rhodamine–phalloidin (Invitrogen, Carlsbad, CA, USA) diluted 1:100 in PB. Nuclei were stained with 4′, 6-diamidino-2-phenylindole (Sigma), diluted 1:200 in PB, for 15 min at room temperature. The slides were rinsed in PB, mounted with polyvinyl alcohol mounting medium with antifade reagent (Sigma) and examined with a confocal microscope. Controls for the experiments consisted of the omission of primary antibody, where no staining was observed. The quantification of fluorescence intensities in five cells per photographed field of five different animals per group were performed with the program ImageJ (National Institutes of Health, Bethesda, MD, USA).

Production of reactive oxygen species by neutrophils

Superoxide anion production by neutrophils

The activity of nicotinamide adenine dinucleotide phosphate-oxidase (NADPH oxidase) in neutrophils was determined by superoxide anion production (O_2^-), which was measured as described by Pick and Mizel.[27] Neutrophils (4×10^5/mL) were incubated with 1 mL of 1.6 mol/L cytochrome *c* (Sigma) solution containing 1 mol/L $CaCl_2$, 0.1 mol/L $MgCl_2$ and 25 ng/mL phorbol 12-myristate 13-acetate (PMA; Sigma) for one hour, at 37°C, in an atmosphere containing 5% CO_2. The same assay was performed in the presence of superoxide dismutase (10 μg, Sigma) and in the absence of PMA, showing the negative and the basal production, respectively. After one hour, the reaction was stopped by immersion of samples in an ice bath for 10 min, the tubes were centrifuged, and the supernatant was read spectrophotometrically at 550 nm, against a blank solution consisting of 1.6 mol/L cytochrome *c* solution. The measurement was performed based on the fact that one mole of O_2^- reduces one mole of cytochrome *c*, and the results were expressed as μmol O_2^- per 4×10^5 neutrophils per hour.

Hydrogen peroxide production by neutrophils

The production of hydrogen peroxide (H_2O_2) was measured as described by Pick and Keisari,[28] modified by Pick and Mizel.[27] Neutrophils (4×10^6/mL) were incubated with 1 mL of 0.28 mmol/L phenol red (Sigma) solution containing 140 mmol/L NaCl, 0.5 mmol/L dextrose and 8.5 U/mL horseradish peroxidase (Sigma). Aliquots of 100 μL (4×10^5 neutrophils) and 40 ng PMA were plated and incubated for one hour, at 37°C, in an atmosphere containing 5% CO_2. The same assay was performed in the absence of PMA to evaluate the basal production of H_2O_2. After this period, the reaction was stopped by the addition of 20 μL of 1 N NaOH. Hydrogen peroxide-dependent phenol red oxidation was measured spectrophotometrically at 620 nm against a blank solution consisting of 0.28 mmol/L phenol red solution. Measurement was performed using a standard curve for H_2O_2, and the results were expressed as nmol H_2O_2 per 4×10^5 neutrophils per hour.

Hypochlorous acid production by neutrophils

The production of hypochlorous acid (HOCl) was measured as described by Dypbukt *et al.*[29] Neutrophils (2×10^5/mL) were incubated with 250 μL of 5 mmol/L taurine (Sigma) solution containing 140 mmol/L NaCl, 10 mmol/L KCl, 0.5 mmol/L $MgCl_2$, 1 mmol/L $CaCl_2$, 1 mg/mL glucose and 25 ng/mL PMA for one hour at 37°C, in an atmosphere containing 5% CO_2. The same assay was performed in the absence of PMA to evaluate the basal production of HOCl. After this period, the reaction was stopped by the addition of 20 μg/mL catalase (Sigma), and the samples were immersed in an ice bath for 10 min. The tubes were then centrifuged; the supernatant was plated, and 50 μL of 2 mmol/L 3,3′,5,5′-tetramethylbenzidine (TMB; Sigma) solution containing 10 μmol KI and 10% dimethylformamide in 400 mmol/L acetate buffer, pH 5.4, were added. After five minutes, the reduction of TMB was measured spectrophotometrically at 650 nm, against a blank solution consisting of 5 mmol/L taurine solution. Measurements were performed using a standard curve for HOCl, and the results were expressed as μmol HOCl per 2×10^5 neutrophils per hour.

Treatments with CdtV and CTX

The effect of CdtV or CTX on phagocytic activity, intracellular signaling involved in this process and the production of reactive oxygen species was evaluated by *in vitro* and *in vivo* assays, using the same experimental design as in our previous studies.[12,14] For *in vitro* experiments, neutrophils were incubated with 1 mL of RPMI 1640 medium (control) or RPMI 1640 medium containing the CdtV (0.25, 0.5, 1.0, 2.5 or 25.0 μg/mL) or CTX (0.04, 0.08, 0.16, 0.32, 0.4 or 4.0 μg/mL) at 37°C and in an atmosphere containing 5% CO_2. After one hour, the cells were centrifuged and prepared for different assays.

For *in vivo* studies, CdtV (0.18 mg/kg in 300 μL of sterile saline) or CTX (0.10 mg/kg in 300 μL of sterile saline) or sterile saline (300 μL, control) was administered subcutaneously two hours, or one, four or 14 days before (pretreatment) or one hour after (post-treatment) the injection of Cg.

The concentrations of CdtV and CTX used in both *in vitro* and *in vivo* assays were based on previous studies[12–14,30] and did not show any sign of cell toxicity as assessed by trypan blue exclusion and the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] test (cell viability >95%). Furthermore, the subcutaneous administration of CdtV and CTX did not cause signs of envenomation in the animals.[31]

Statistical analysis

Statistical analyses of the differences between the groups were performed according to Glantz,[32] using GraphPad InStat software, version 3.01 (GraphPad Software Inc., San Diego, CA, USA). One-way analysis of variance followed by Tukey's test was used for multiple comparisons (all pairs of groups) in assays of *in vitro* phagocytosis. To analyze the other assays, one-way analysis of variance followed by Bonferroni's test were used for multiple comparisons against a single control, or unpaired Student's *t*-test were used to compare two groups. Differences with $P < 0.05$ were considered statistically significant. Results are presented as the mean ± standard error of mean of values obtained with the indicated number of animals.

Results

Effect of CdtV on phagocytic activity of neutrophils

In vitro

CdtV, at 0.25, 0.5, 1.0 and 2.5 μg/mL, caused a significant inhibition of the phagocytic activity of neutrophils, when compared with the control, but no statistical differences were observed between these concentrations. However, CdtV at 25.0 μg/mL did not inhibit this activity (Figure 1a – $F[5,24] = 18.82$, $P < 0.0001$).

To evaluate the contribution of CTX to the inhibitory effect of CdtV, neutrophils were incubated with this toxin. CTX at all concentrations (0.04, 0.08, 0.16, 0.32, 0.4 and 4.0 μg/mL) caused a significant reduction of phagocytosis when compared with the control. However, only CTX at 0.4 μg/mL was statistically different from the other concentrations (Figure 1b – $F[6,28] = 9.33$, $P < 0.0001$).

To confirm that only CTX contributed to this inhibitory effect of crude venom, the phagocytosis assay was carried out with the remaining pooled material (peaks 1 and 3) obtained during the CTX purification process; i.e. the other unpurified venom toxins. The incubation of neutrophils with either peak did not alter the phagocytic activity of these cells (data not shown).

In vivo

The subcutaneous injection of CdtV or CTX also reduced the percentage of phagocytosis when compared with the control. The decrease in phagocytic activity in animals pretreated with CdtV was 42% at two hours, 43% at one day, 51% at four days and 23% at 14 days when compared with the respective control (Figure 2a – 2 h: $t = 3.91$, $P = 0.0045$; 1 day: $t = 4.91$, $P = 0.0012$; 4 d: $t = 6.69$, $P = 0.0002$; 14 d: $t = 3.98$, $P = 0.0040$). Post-treatment with CdtV, one hour after Cg, also inhibited the phagocytic activity of neutrophils by 39% (Cg + saline: 142 ± 8.22, Cg + CdtV: 87.8 ± 4.8; $t = 5.69$, $P = 0.0005$).

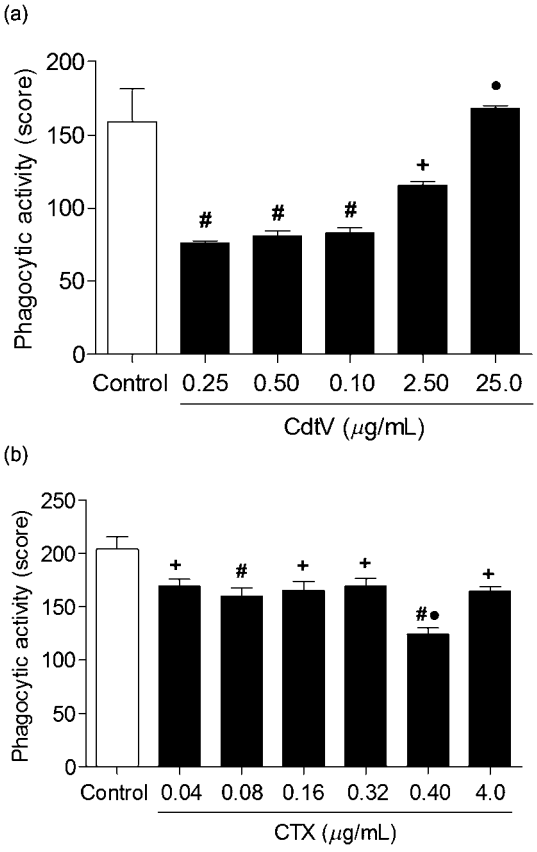

Figure 1 *In vitro* effect of *Crotalus durissus terrificus* venom (CdtV) and crotoxin (CTX) on phagocytosis by neutrophils. Neutrophils were obtained from the peritoneal cavity, four hours after the intraperitoneal administration of carrageenan (4.5 mg/kg). Cells (1×10^6/mL) were incubated for one hour with different concentrations of CdtV (0.25, 0.5, 1.0, 2.5 or 25.0 μg/mL) (a), CTX (0.04, 0.08, 0.16, 0.32, 0.4 or 4.0 μg/mL) (b) or with RPMI 1640 medium (control). Opsonized zymosan particles (5×10^6/mL) were used as the phagocytic stimulus. A total of 100 cells were counted and the phagocytic activity determined. Values are expressed as the mean ± SEM of five rats per group. $^{\#}P < 0.01$, $^{+}P < 0.05$ when compared with control, $^{\bullet}P < 0.01$ when compared with all concentrations of CdtV or CTX (analysis of variance/Tukey's test)

A decrease was also observed in phagocytosis by neutrophils in animals pretreated with a single injection of CTX at two hours or one, four or 14 days before the administration of Cg. This inhibition was 24% with two hours, 31% with one day, 25% with four days, and 18% with 14 days (Figure 2b – 2 h: $t = 8.34$, $P < 0.0001$; 1 d: $t = 7.61$, $P < 0.0001$; 4 d: $t = 4.78$, $P = 0.0014$; 14 d: $t = 7.82$, $P < 0.0001$). Post-treatment, one hour after Cg, also inhibited the phagocytic activity of neutrophils by 35% (Cg + saline: 130 ± 1.89, Cg + CTX: 198.4 ± 4.06; $t = 15.23$, $P < 0.0001$).

Effect of CdtV and CTX on tyrosine phosphorylation and actin polymerization during phagocytosis by neutrophils

To characterize the inhibitory effect of CdtV and CTX on phagocytosis by neutrophils, we investigated their action

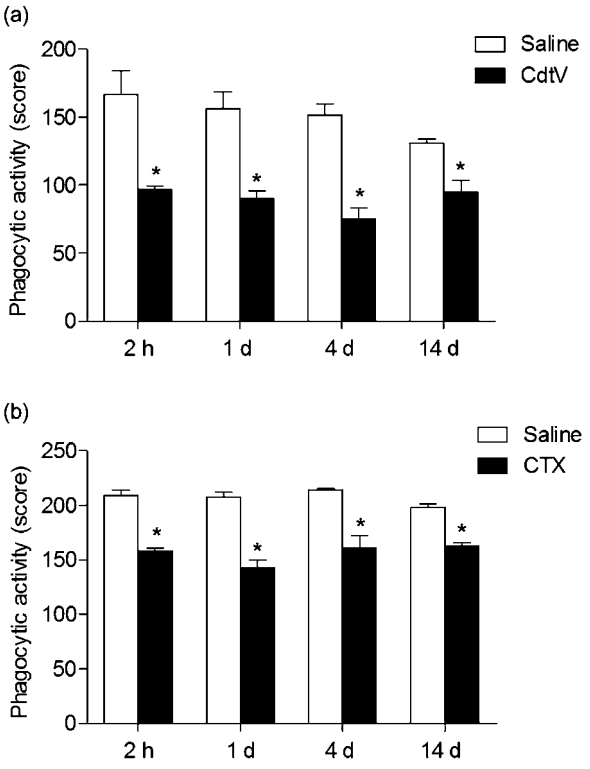

Figure 2 *In vivo* effect of *Crotalus durissus terrificus* venom (CdtV) and crotoxin (CTX) on phagocytosis by neutrophils. Neutrophils were obtained from the peritoneal cavity, four hours after the intraperitoneal administration of carrageenan (Cg) (4.5 mg/kg). CdtV (0.18 mg/kg) (a), CTX (0.1 mg/kg) (b) or saline (control) were subcutaneously injected two hours, one, four or 14 d before the intraperitoneal injection of Cg. Opsonized zymosan particles (5×10^6/mL) were used as the phagocytic stimulus. A total of 100 cells were counted and the phagocytic activity determined. Values are expressed as mean $\pm$ SEM of five rats per group. $^*P < 0.01$ when compared with the respective control (Student's *t*-test)

on tyrosine phosphorylation and actin polymerization in nascent (phagocytosis for 5 min) and mature phagosomes (phagocytosis for 15 min).

Neutrophils incubated with CdtV (0.5 μg/mL) or CTX (0.08 μg/mL) and tested for phagocytosis of opsonized zymosan particles for five minutes showed a significant reduction in staining of phosphotyrosine (CdtV: 89%, CTX: 98%) and F-actin (CdtV: 71%, CTX: 80%) when compared with the control. Neutrophils incubated with only RPMI 1640 medium (control) had intense staining of phosphotyrosine and F-actin in regions of the plasma membrane involved in phagosome formation (Figure 3 – $F[5,24] = 259.35$, $P < 0.0001$).

After 15 min of phagocytosis, neutrophils incubated with CdtV (0.5 μg/mL) or CTX (0.08 μg/mL) showed a significant reduction in F-actin staining (Cg + saline: 32.23 ± 2.18, Cg + CdtV: 9.52 ± 0.69, Cg + CTX: 4.49 ± 1.08, $F[2,12] = 170.16$, $P < 0.0001$) when compared with the control. Neutrophils incubated with only RPMI 1640 medium (control) showed weak staining of phosphotyrosine and intense staining of F-actin around the phagosomes formed.

In *in vivo* experiments, pretreatment of the animals with CdtV (0.18 mg/kg) or CTX (0.1 mg/kg), two hours (Figure 4 – $F[5,24] = 59.93$, $P < 0.0001$), 4 d (Figure 5 – $F[5,24] = 90.16$, $P < 0.0001$) or 14 d (Figure 6 – $F[5,24] = 20.81$, $P < 0.0001$) before intraperitoneal administration of Cg strongly inhibited tyrosine phosphorylation (2 h – CdtV: 86%, CTX: 85%; 4 d – CdtV: 68%, CTX: 78%; 14 d – CdtV: 70%, CTX: 74%) and actin polymerization (2 h – CdtV: 83%, CTX: 90%; 4 d – CdtV: 84%, CTX: 86%; 14 d – CdtV: 49%, CTX: 49%) in nascent phagosomes (5 min of phagocytosis), when compared with animals pretreated with only saline (control).

Effect of CdtV and CTX on production of reactive oxygen species by neutrophils

The incubation of neutrophils with different concentrations of CdtV (0.25, 0.5 or 1.0 μg/mL) or CTX (0.02, 0.04 or 0.08 μg/mL) did not alter the production of superoxide anion, hydrogen peroxide or hypochlorous acid by neutrophils. Statistical differences were observed between control and control + PMA (Figure 7a – $F[11,48] = 32.82$, $P < 0.0001$; Figure 7b – $F[11,48] = 16.42$, $P < 0.0001$; Figure 7c – $F[11,48] = 52.97$, $P < 0.0001$; Figure 7d – $F[11,48] = 80.12$, $P < 0.0001$; Figure 7e – $F[11,48] = 14.90$, $P < 0.0001$; Figure 7f – $F[11,48] = 12.71$, $P < 0.0001$). Similarly, pretreatment of animals with CdtV (0.18 mg/kg) or CTX (0.1 mg/kg), two hours before Cg, did not alter the production of these reactive oxygen species by neutrophils. However, statistical differences were observed between controls (saline + Cg and saline + Cg + PMA) (Table 1 – O_2^-: $F[5,24] = 24.76$, $P < 0.0001$; H_2O_2: $F[5,24] = 106.44$, $P < 0.0001$; HOCl: $F[5,24] = 16.98$, $P < 0.0001$).

Discussion

The venom of *C. durissus terrificus* has long-lasting anti-inflammatory properties and modulates some of the functions of macrophages.

We demonstrated here that CdtV inhibits *in vitro* and *in vivo* the phagocytosis of serum-opsonized zymosan particles by inflammatory neutrophils obtained in a rat model of peritonitis induced by Cg.

The data also showed that, like crude venom, CTX inhibited the phagocytic activity of neutrophils; on the other hand, the remaining pooled material obtained during the CTX purification process[22] did not affect this activity. These results demonstrate that CTX is the component of the venom responsible for the inhibitory effect on phagocytosis by neutrophils. As confirmed by trypan blue exclusion and MTT assay, the inhibitory effect of CdtV and CTX showed no relation to cytotoxicity. This effect was similar for all concentrations evaluated, since the incubation of cells with concentrations varying 2- to 10-fold did not change the inhibition induced by the venom. However, for CdtV concentrations 100-fold, the inhibitory effect was no longer observed. These results are in agreement with our previous results, where the same concentrations of CdtV inhibited phagocytosis by macrophages without altering cell viability. Furthermore, these results re-inforce the findings in the literature that show that CTX accounts for the inhibitory effect of CdtV on phagocytosis by

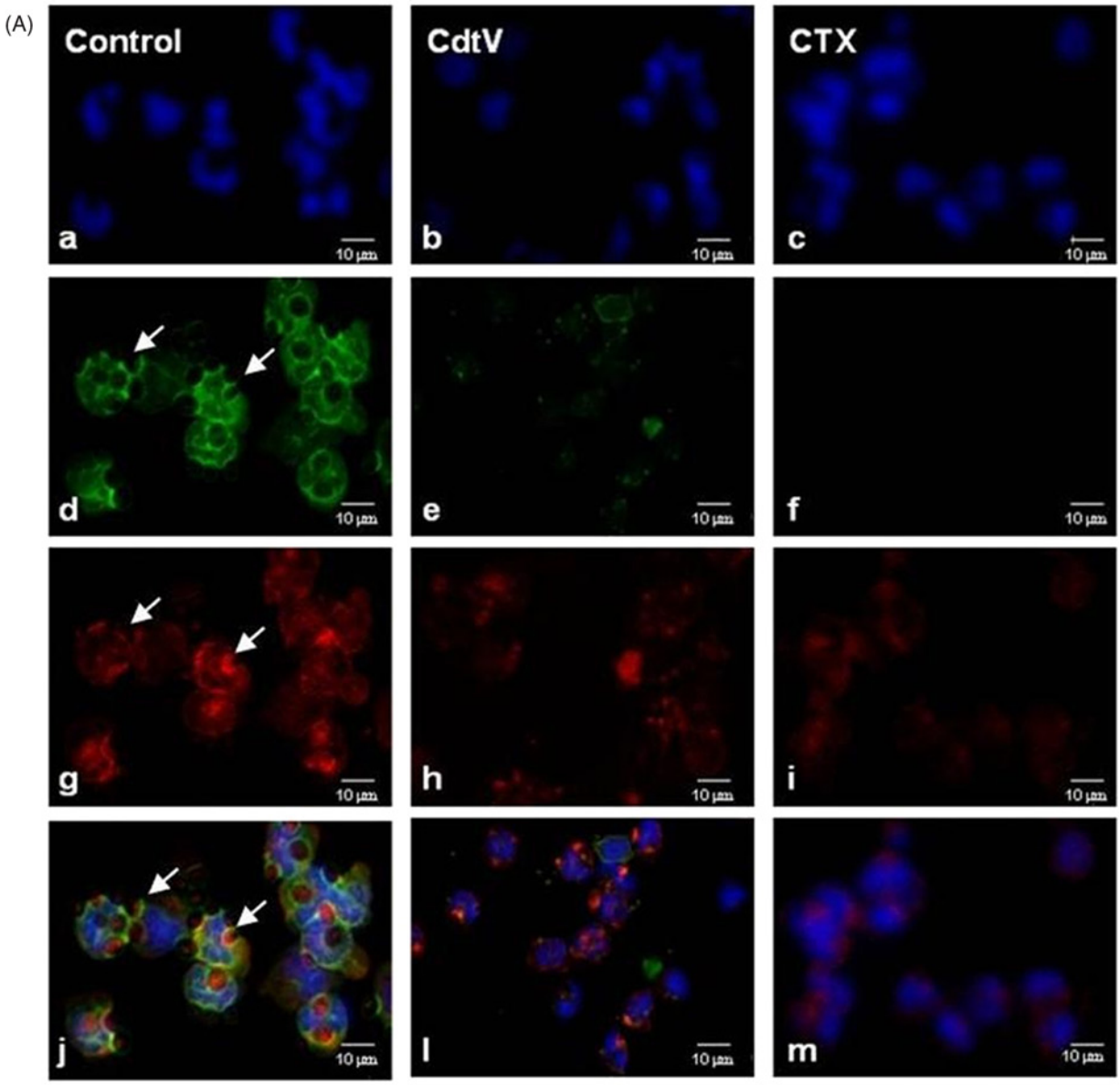

Figure 3 *In vitro* effect of *Crotalus durissus terrificus* venom (CdtV) and crotoxin (CTX) on tyrosine phosphorylation and actin polymerization during phagocytosis. Neutrophils were obtained from the peritoneal cavity, four hours after the intraperitoneal administration of carrageenan (4.5 mg/kg). Cells (1×10^6/mL) were incubated for one hour with Cdtv (0.5 μg/mL), CTX (0.08 μg/mL) or RPMI 1640 medium (control). (A) After incubation, neutrophils were allowed to phagocytize opsonized zymosan particles for five minutes, and phosphotyrosine (anti-PTry 1:100 + fluorescein isothiocyanate [FITC] 1:200) and F-actin (phalloidin + rhodamine 1:100) were then detected by immunocytochemical assays. a, b and c represent the labeling of nuclei (6-diamidino-2-phenylindole); d, e and f represent the labeling of phosphotyrosine (FITC); g, h and i represent the labeling of F-actin (rhodamine); and j, l and m represent the overlapping images. (→) Nascent phagosome containing zymosan particle. (B) Quantification of fluorescence intensities was performed in five cells per photographed field by the program ImageJ, and the results are expressed as the mean ± SEM of five rats per group. $^{\#}P < 0.01$ when compared with control (analysis of variance/Bonferroni's test). (A color version of this figure is available in the online journal)

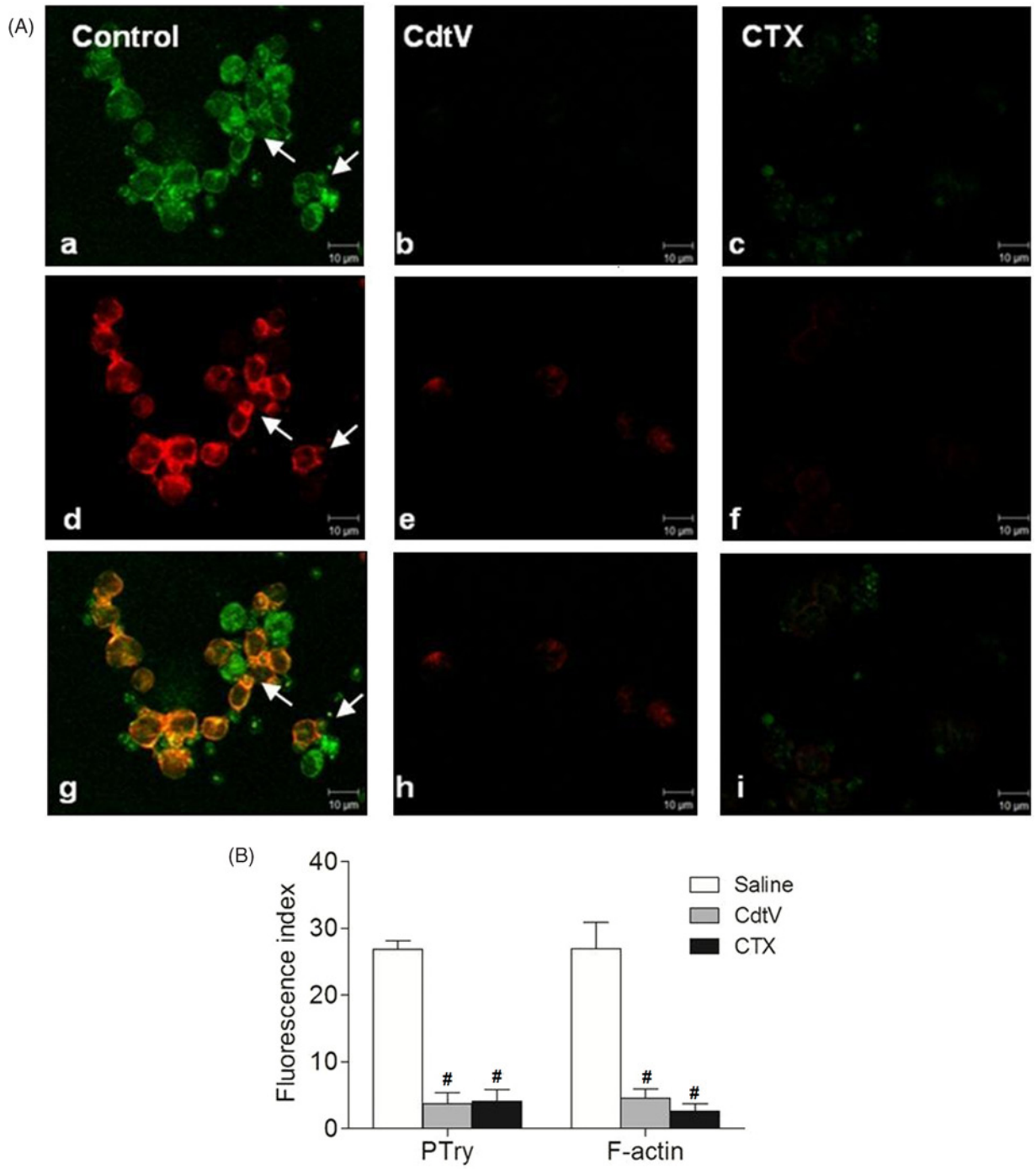

Figure 4 *In vivo* effect of *Crotalus durissus terrificus* venom (CdtV) and crotoxin (CTX) on tyrosine phosphorylation and actin polymerization during phagocytosis – pretreatment for two hours. CdtV (0.18 mg/kg), CTX (0.1 mg/kg) or saline (control) were subcutaneously injected two hours before the intraperitoneal injection of carrageenan (4.5 mg/kg). (A) Neutrophils (1×10^6/mL) collected from the peritoneal exudate were allowed to phagocytize opsonized zymosan particles for five minutes and phosphotyrosine (anti-PTry 1:100 + fluorescein isothiocyanate [FITC] 1:200) and F-actin (phalloidin + rhodamine 1:100) were then detected by immunocytochemical assays. a, b and c represent the labeling of phosphotyrosine (FITC); d, e and f represent the labeling of F-actin (rhodamine); and g, h and i represent the overlapping images. (→) Nascent phagosome containing zymosan particle. (B) Quantification of fluorescence intensities was performed in five cells per photographed field by the program ImageJ, and the results are expressed as the mean ± SEM of five rats per group. $^{\#}P < 0.01$ when compared with control (analysis of variance/Bonferroni's test). (A color version of this figure is available in the online journal)

macrophages and for the anti-inflammatory action of this venom.[12,14]

VCdt and CTX not only have a direct inhibitory effect on the phagocytic activity of neutrophils, but also a long-lasting systemic effect that changes the basic function of this cell, since the same inhibitory effect was observed *in vivo* when a single injection of crude venom or CTX was subcutaneously administered two hours, or one, four or 14 days before or one hour after Cg. These observations are consistent with previous data that showed a long-lasting inhibitory effect of CdtV and CTX on the process of phagocytosis by macrophages and

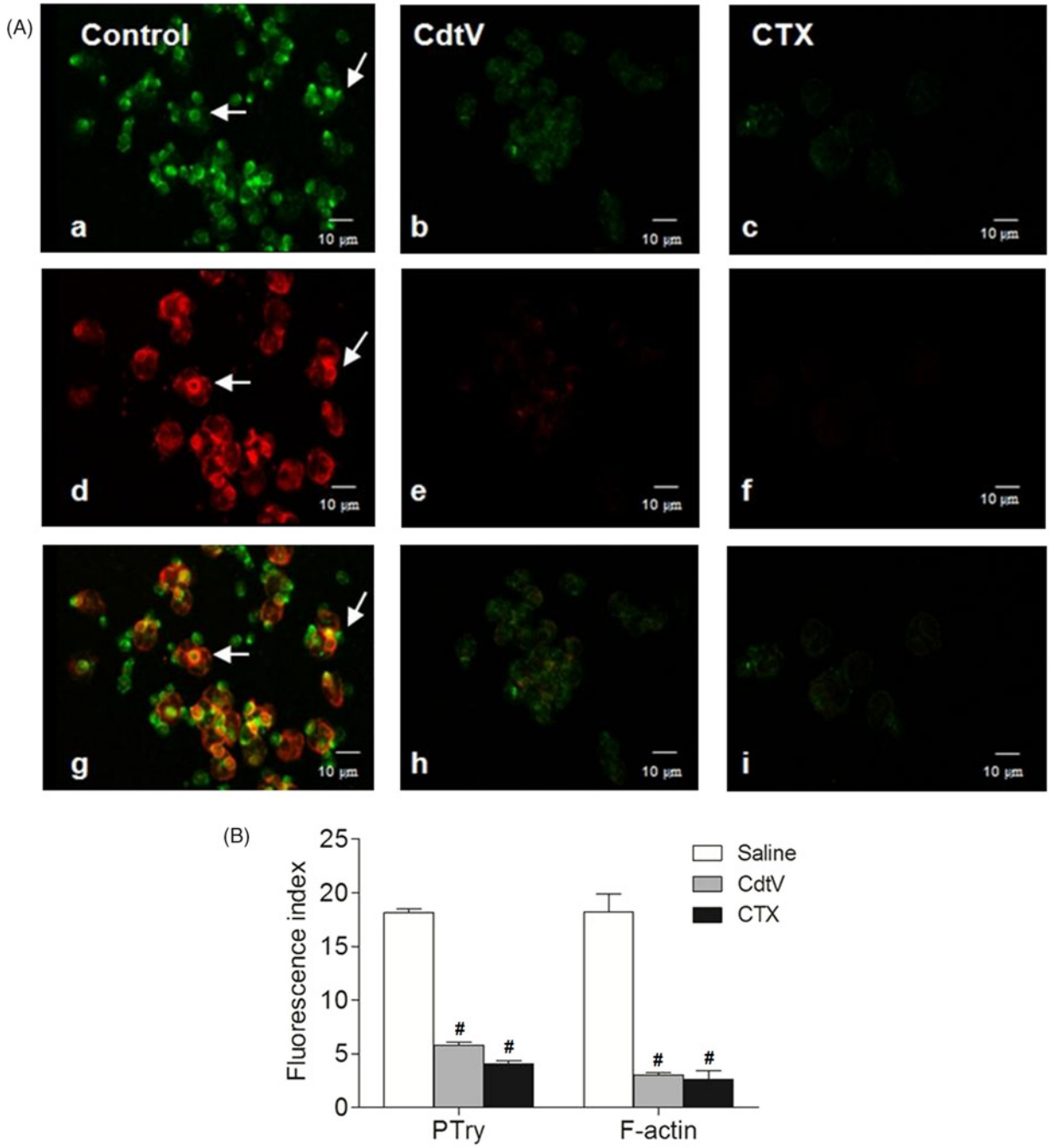

Figure 5 *In vivo* effect of *Crotalus durissus terrificus* venom (CdtV) and crotoxin (CTX) on tyrosine phosphorylation and actin polymerization during phagocytosis – pretreatment for four days. CdtV (0.18 mg/kg), CTX (0.1 mg/kg) or saline (control) were subcutaneously injected four days before the intraperitoneal injection of carrageenan (4.5 mg/kg). (A) Neutrophils (1×10^6/mL) collected from the peritoneal exudate were allowed to phagocytize opsonized zymosan particles for five minutes and phosphotyrosine (anti-PTry 1:100 + fluorescein isothiocyanate [FITC] 1:200) and F-actin (phalloidin + rhodamine 1:100) were detected by immunocytochemical assays. a, b and c represent the labeling of phosphotyrosine (FITC); d, e and f represent the labeling of F-actin (rhodamine); and g, h and i represent the overlapping images. (→) Nascent phagosome containing zymosan particle. (B) Quantification of fluorescence intensities was performed in five cells per photographed field by the program ImageJ, and the results are expressed as the mean ± SEM of five rats per group. $^{\#}P < 0.01$ when compared with control (analysis of variance/Bonferroni's test). (A color version of this figure is available in the online journal)

inflammatory response.[8,9] A pharmacokinetic study showed that approximately 95% of the toxin is eliminated in 24 h, and thus, it is difficult to explain the long-lasting effect of CTX on the function of inflammatory cells.[33] Neutrophils and macrophages arise from a common bone marrow precursor; these phagocytes originate from stem cells that differentiate through common myeloid progenitors and granulocyte/macrophage progenitors.[34,35] Moreover, these phagocytes have complementary characteristics and cooperate as effectors and modulators of the immune response.[36] Thus, we suggest that the long-lasting downregulation induced by CTX in these phagocytes could be due to the action of this toxin on progenitor cells. This hyphothesis will be investigated.

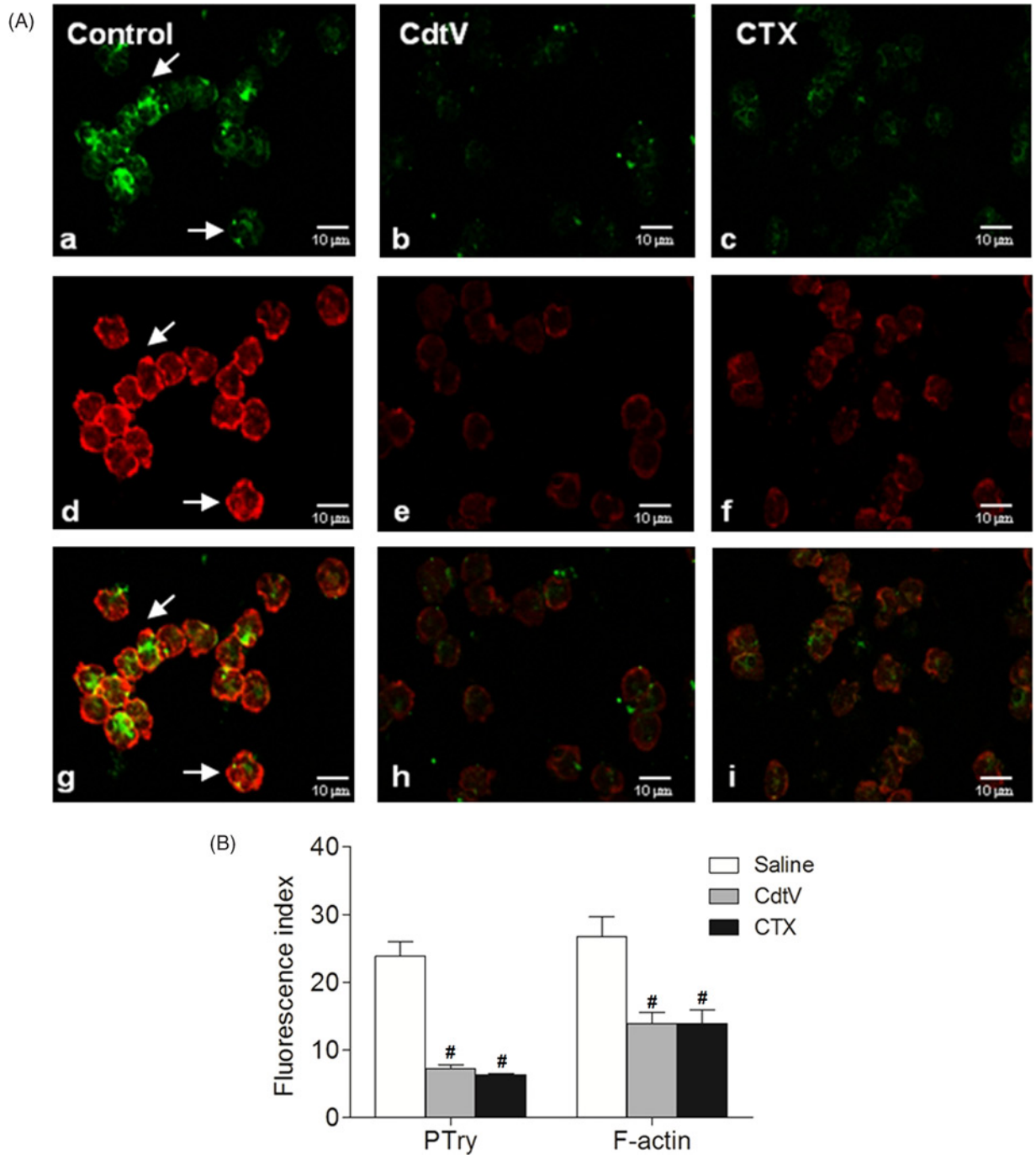

Figure 6 *In vivo* effect of *Crotalus durissus terrificus* venom (CdtV) and crotoxin on tyrosine phosphorylation and actin polymerization during phagocytosis – pretreatment for 14 d. CdtV (0.18 mg/kg), CTX (0.1 mg/kg) or saline (control) were subcutaneously injected 14 d before the intraperitoneal injection of carrageenan (4.5 mg/kg). (A) Neutrophils (1×10^6/mL) collected from the peritoneal exudate were allowed to phagocytize opsonized zymosan particles for five minutes and phosphotyrosine (anti-PTry 1:100 + fluorescein isothiocyanate [FITC] 1:200) and F-actin (phalloidin + rhodamine 1:100) were then detected by immunocytochemical assays. a, b and c represent the labeling of phosphotyrosine (FITC); d, e and f represent the labeling of F-actin (rhodamine); and g, h and i represent the overlapping images. (→) Nascent phagosome containing zymosan particle. (B) Quantification of fluorescence intensities was performed in five cells per photographed field by the program ImageJ, and the results are expressed as the mean ± SEM of five rats per group. $^{\#}P < 0.01$ when compared with control (analysis of variance/Bonferroni's test). (A color version of this figure is available in the online journal)

To further study the inhibitory effect of CTX on the process of phagocytosis by neutrophils, we examined whether the CTX could change tyrosine phosphorylation and actin polymerization in nascent and mature phagosomes. The results showed that CTX, *in vitro* or *in vivo*, induced a decrease in tyrosine phosphorylation and polymerization of F-actin in neutrophils during five minutes of phagocytosis of opsonized zymosan particles (nascent phagosomes). However, in neutrophils during 15 min of phagocytosis (mature phagosome), tyrosine phosphorylation was not observed in cells incubated with RPMI 1640 medium only or CTX, since the process of

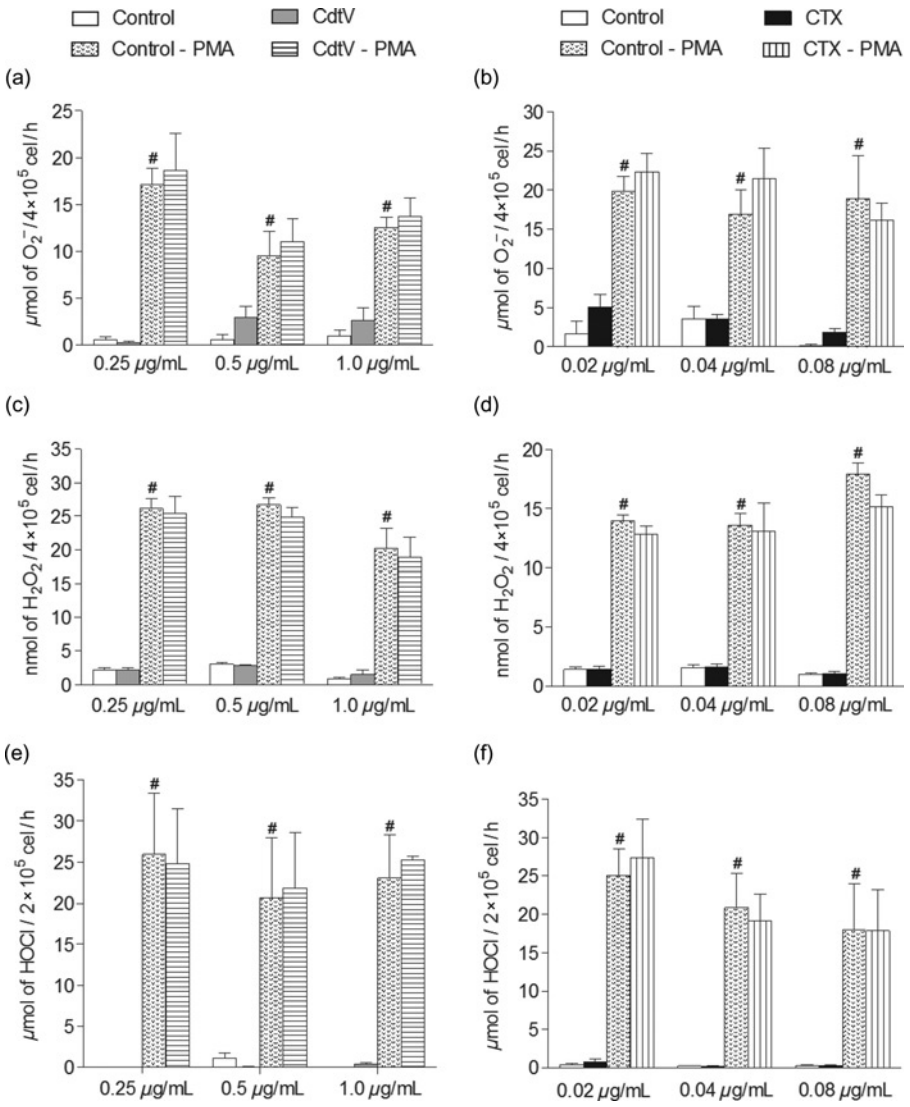

Figure 7 *In vitro* effect of *Crotalus durissus terrificus* venom (CdtV) and crotoxin (CTX) on production of reactive oxygen species by neutrophils. Neutrophils were obtained from peritoneal cavity, four hours after the intraperitoneal administration of carrageenan (4.5 mg/kg). Cells (1×10^6/mL) were incubated for one hour with different concentrations of CdtV (0.25, 0.5 or 1.0 μg/mL), CTX (0.02, 0.04 or 0.08 μg/mL) or with RPMI 1640 medium (control). After incubation, the production of superoxide anion (O_2^-; e, b), hydrogen peroxide (H_2O_2; c, d) or hypochlorous acid (HOCl; e, f) was evaluated in the presence or absence of phorbol 12-myristate 13-acetate (PMA). The results are expressed as the mean $\pm$ SEM of five rats per group. $^{\#}P < 0.01$ when compared with the respective control (analysis of variance/Bonferroni's test)

phagocytosis in neutrophils occurs very fast and immediately after binding the particle with the receptor.[37,38] Taking this into account, in mature phagosomes, it was only possible to evaluate the *in vitro* effect of CTX on actin filaments. As observed for the nascent phagosomes, CTX inhibited actin polymerization in mature phagosomes. This effect of CTX on neutrophils differs from that observed in previous studies, which showed that CTX increases actin polymerization during phagocytosis by macrophages.[15] However, it is important to recall that the maturation of a nascent phagosome differs between neutrophils and macrophages.[39] This may explain, at least in part, the observed differences between the effect of CTX on phagocytosis by macrophages and neutrophils.

Rho protein has a significant role in the regulation of actin polymerization in phagocytosis mediated by the receptors CR1/CR3, and tyrosine phosphorylation is an important event.[40] We therefore suggest that the inhibition of tyrosine phosphorylation induced by CTX may lead to changes in the activation of these signaling proteins, such as RhoA, and thus inhibits phagocytosis; however, further studies are needed to understand the intracellular mechanisms involved in the inhibitory effect of CTX in the process of phagocytosis by neutrophils.

Since phagocytosis results in the release of microbicidal substances and NADPH oxidase activation with subsequent generation of reactive oxygen species, we investigated the effect of CTX on production of reactive oxygen species by neutrophils.

The results showed that CdtV and CTX did not change microbicidal activity (data not shown) and the production of reactive oxygen species by neutrophils; in contrast to that observed for macrophages in which CdtV stimulates candidacidal activity by hydrogen peroxide

Table 1 *In vivo* effect of *Crotalus durissus terrificus* venom (CdtV) and crotoxin (CTX) on the production of reactive oxygen species by neutrophils

Treatment	μmol O_2^-/ 4×10^5cel/h	nmol H_2O_2/ 4×10^5cel/h	μmol HOCl/ 2×10^5cel/h
Saline + Cg	3.81 ± 1.08	1.03 ± 0.14	0.61 ± 0.02
CdtV + Cg	1.78 ± 1.05	1.22 ± 0.06	0.55 ± 0.02
CTX + Cg	1.79 ± 0.63	1.48 ± 0.41	0.47 ± 0.05
Saline + Cg + PMA	10.57 ± 1.15*	30.22 ± 3.33*	28.18 ± 5.78*
CdtV + Cg + PMA	10.11 ± 1.46	25.42 ± 1.79	25.47 ± 6.17
CTX + Cg + PMA	12.84 ± 1.89	30.76 ± 2.73	25.50 ± 4.96

Neutrophils were obtained from the peritoneal cavity of rats four hours after the administration of Cg. The production of reactive oxygen species was determined in the absence and in the presence of PMA. CdtV (0.18 mg/kg), CTX (0.10 mg/kg) or saline (control) was subcutaneously injected two hours before Cg. The results are expressed as the mean ± SEM of five rats per group.

Cg, carrageenan; PMA, phorbol 12-myristate 13-acetate; O_2^-, superoxide anion; H_2O_2, hydrogen peroxide; HOCl, hypochlorous acid

*$P < 0.01$ when compared with the respective control (analysis of variance/Bonferroni's test)

overproduction.[13] Despite the association between the process of phagocytosis and microbicidal activity, Rho protein is not involved in the activation of NADPH oxidase in response to PMA (stimulatory agent used in our assays), where Rac2 is the main protein involved in this activation.[41,42] Thus, the differences between the signaling pathways involved in phagocytosis and the generation of reactive oxygen species could explain the inhibitory effect of CTX, particularly with regards to phagocytosis.

In conclusion, this study characterizes for the first time the actions of CdtV on neutrophils and demonstrates that CTX induces a long-lasting inhibition of tyrosine phosphorylation, actin polymerization and consequently phagocytosis. Thus, considering that neutrophils have a pivotal role in inflammation, this study also contributes to the elucidation of the mechanisms involved in the anti-inflammatory action of CTX. Moreover, there are many drugs that are able to inhibit neutrophil responses, acting by impairing some of the different steps of these transduction pathways. A major limitation in their use as therapeutic agents for the treatment of inflammation-related diseases is that these drugs are not selective, since other cellular responses could be inhibited at the same time. Accordingly, CTX may represent a potential natural product in controlling inflammatory disease, since a single dose induces a long-lasting effect on intracellular signaling involved in phagocytosis by neutrophils.

Author contributions: All authors participated in the design, interpretation of the studies, analysis of the data and review of the manuscript. MSD-C supplied crotoxin; TSL developed the research; and MCC designed and coordinated the research.

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