Toxicology in Vitro 25 (2011) 358–367

Contents lists available at ScienceDirect

Toxicology in Vitro

journal homepage: www.elsevier.com/locate/toxinvit

Specific activation of human neutrophils by scorpion venom: A flow cytometry assessment

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ARTICLE INFO

Article history:
Received 23 February 2010
Accepted 12 October 2010
Available online 20 October 2010

Keywords:
Tityus
Venezuela
Neutrophils
Eosinophils
Staurosporine
Phorbol ester
ROS
Scorpion venom
Tityus zulianus
Tityus discrepans

ABSTRACT

Acute lung injury following envenomation by *Tityus* scorpion species is due in part to activation of the inflammatory response leading to release of cytotoxic leukocyte-derived products, including cytokines and possibly reactive oxygen species (ROS). *Tityus zulianus* envenomation in Venezuela produces cardiorespiratory complications and death by lung injury whereas stings by *Tityus discrepans* produce mainly gastrointestinal and pancreatic alterations. To ascertain the role played by granulocytes in the envenomation by *T. zulianus* (TzV) and *T. discrepans* (TdV), human peripheral blood neutrophils, eosinophils, and monocytes were exposed to scorpion venoms (0.001–5 μg/mL) and the kinetics (5–15 min) of peroxide production determined by flow cytometry, using 2′,7′-dichlorodihydrofluorescein diacetate (succinimidyl ester) as a fluorescent substrate. TzV induced a significantly ($p < 0.01$) more potent increase in peroxide production in neutrophils (for 5 and 10 min of incubation), and to a lesser extent in monocytes (5–15 min), compared to TdV. TzV induced necrosis in neutrophils at doses higher than 5 μg/mL. No effect was observed on eosinophils, suggesting that TzV specifically targets neutrophil intracellular ROS production. The TzV-stimulated pathway is protein kinase C-dependent because it was almost completely (>90%) abolished by staurosporine. The stimulatory effect is associated with the lowest molecular mass venom peptides as gel filtration fractions TzII and TzIII significantly enhanced peroxide production. The combined used of the intracellular ROS agonist, phorbol myristate acetate (PMA), and TzV produced a modest but significant increase in peroxide production suggesting the possibility of overlapping signaling cascades amongst PMA and TzV. Up-regulation of intracellular neutrophil ROS production may be an important *in vivo* target for TzV which could have a role to play in the cardiorespiratory complications elicited after envenomation by this species.

1. Introduction

Scorpion envenomation is a global public health problem particularly in tropical and subtropical areas (Chippaux and Goyffon, 2008). Northern South America (encompassing Venezuela, Colombia, Ecuador and the Guianas) is one of the hyperendemic areas of scorpionism in the world, with over 100 cases per 100,000 inhabitants. *Tityus* is the scorpion genus accounted for most severe and fatal cases of scorpion sting in this area (Borges and De Sousa, 2006; Daisley et al., 1999; De Sousa et al., 2000).

Recent phylogenetic and antigenicity evidence suggest that in parallel to the strong biogeographic structuring of *Tityus* species in this area, toxinological partitioning exists, related to compositional and functional differences in the venom of medically important species (Borges et al., 2010a,b). In this respect, clinical data indicate that the *Tityus* envenoming syndrome depends on the scorpion species involved in the accident. For example, stings by *Tityus zulianus* (Zulia, Mérida, and Táchira States, western Venezuela) often produce respiratory arrest and death by pulmonary oedema, whereas envenomation by *Tityus discrepans* (north-central Venezuela) mainly causes pancreatic and gastrointestinal disorders. These two species are responsible for most casualties in Venezuelaś western and north-central ranges, respectively (Borges and De Sousa, 2006). Even though the mechanisms responsible for the

Abbreviations: FITC, fluorescein isothiocyanate; H_2DCFDA-SE, 2′,7′-dichlorodihydrofluorescein diacetate, succinimidyl ester; Na_v, voltage-sensitive sodium channel; NOX, NADPH oxidase; PAF, platelet activating factor; PMA, phorbol 12-myristate 13-acetate; PKC, protein kinase C; ROS, reactive oxygen species; TdV, *Tityus discrepans* venom; TzV, *Tityus zulianus* venom.

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doi:10.1016/j.tiv.2010.10.009

T. zulianus-induced severe cardiorespiratory complications have yet to be fully elucidated, a massive cathecolaminergic discharge, resulting from the scorpion toxin action on the sympathetic nervous system, has been implicated (Mazzei de Dávila et al., 2002). In addition, Borges et al. (2004) have also found that the pancreas is significantly more affected after *T. zulianus* experimental envenomation compared with *T. discrepans*, probably as a result of cholinergic hyperstimulation. Since inflammatory mediators released from the affected pancreas can lead to distant organ damage including the lung (Zhou et al., 2010), the pancreas exocrine alterations elicited by *T. zulianus* may have a role to play in the increased cardiorespiratory alterations produced by this venom. Transcriptomic and mass spectral analyses have also revealed differences between venoms from *T. zulianus* (TzV) and *T. discrepans* (TdV) (Borges et al., 2006).

Of paramount importance in severe *Tityus* envenomation is the syndrome of acute lung injury, which is initiated by a variety of systemic insults leading to diffuse damage to the pulmonary parenchyma. The first clinically recognizable consequences of this injury are in large part attributable to an increase in the permeability of the alveolar-capillary membrane with subsequent pulmonary edema (Coelho et al., 2007; Paneque Peres et al., 2009). Damage to the lung is the consequence, at least in part, of an activation of the acute inflammatory response leading to release of cytotoxic leukocyte-derived products such as proteolytic enzymes, cationic proteins, eicosanoids, cytokines and possibly reactive oxygen species (ROS). In this sense, previous studies have demonstrated that venom from the Brazilian scorpion *Tityus serrulatus* increases the serum levels of IL-1, IL-6, INF-γ, and granulocyte–macrophage colony-stimulating factor in patients with severe shock and pulmonary edema (Fukuhara et al., 2003). Moreover, it has been shown that experimental models of scorpion envenomation presented an increase in serum levels of IL-1, IL-6, IFN-γ and nitric oxide (Petricevich and Peña, 2002).

Recent evidence suggests that *T. serrulatus* venom possesses significant immunomodulatory activities capable of stimulating immune functions *ex vivo*, probably through the activation of voltage-gated ion channels in immune cells (Petricevich et al., 2008). For example, venom incubation of rat peritoneal macrophages produces an increased production of IL-6 and IFN-γ in a dose-dependent manner and also induced an elevation in hydrogen peroxide release (Petricevich, 2002). Also, a dramatic blood neutrophilia, mediated through the activation of platelet activating factor (PAF) receptors, has been associated with *T. serrulatus* envenomation which may contribute to the development of lung injury in children (Borges et al., 2000). Strong neutrophil infiltration, predominantly in the lungs, has been observed after subcutaneous injection with TdV in rams (D'Suze et al., 2004).

In view of the possibility that cytotoxic leukocyte-derived products, including ROS, could be involved in the mechanism responsible for severe lung injury associated with the envenomation by *Tityus* in Venezuela, we wished to compare peroxide production in human leukocytes (e.g., neutrophils, eosinophils, and monocytes) exposed to TzV and TdV, the two species responsible for severe scorpionism in the country. It was found that the more potent effect of TzV (as opposed to TdV) on peroxide production was specific to neutrophils and was associated with the venom lowest molecular mass peptides. To our knowledge this is the first report on the specific activation by *Tityus* scorpion venoms of neutrophil signaling cascades involved in ROS generation. The data reinforce the notion that functional differences exist amongst *Tityus* venoms which can account for the differential clinical manifestations observed in envenomated humans.

2. Materials and methods

2.1. Scorpion venoms

Scorpions were collected at night using ultraviolet lamps. Adult *T. zulianus* scorpions ($n = 10$) were collected around Santa Cruz de Mora (8° 22′ N, 71° 43′ W), western Mérida State, Venezuela. Adult *T. discrepans* scorpions ($n = 10$) were collected near San Antonio de Los Altos (10° 20′ N, 67° 45′ W), Miranda State, central Venezuela. The arachnids were kept in captivity at the Institute of Experimental Medicine, Caracas, as described by Borges et al. (2006). Venom was pooled from at least 20 scorpions and obtained by manual stimulation of the telson (the last caudal segment) by forcing the animals to sting repeatedly onto Parafilm® sheets. Venom was subsequently lyophilized at −50 °C and 80 mbar of pressure and reconstituted with doubly distilled water before use at 0.5 mL/mg. Protein concentration was determined according to Lowry et al. (1951).

2.2. Scorpion venom fractionation and mass spectral analyses

TzV (5 mg total protein) was fractionated by gel filtration chromatography using Sephadex G-50 columns (0.9 × 70 cm) eluted at a flow rate of 1 mL/h. Elution of fractions was recorded at 280 nm. SDS–PAGE on 15% acrylamide gels was used to inspect fraction composition according to Borges et al. (2006). Composition of fractions TzI, TzII, and TzIII was further analyzed by matrix-assisted time-of-flight mass spectrometry (MALDI-TOF) basically as described previously (Borges et al., 2006) in a Biflex III MALDI-TOF MS (Bruker, FRG).

2.3. Cell separation

Human leukocyte cells were isolated from heparinized venous blood (10 U/mL), obtained from healthy adult donors, following informed consent, in accordance with the institutional protocols from the Ethical Committee, Instituto de Inmunología, Central University of Venezuela. Donors were healthy adult males and females over the age of 18, with normal hematological parameters who did not have infectious or chronic diseases. Mononuclear cells were separated by Bøyum's Ficoll-Hypaque method (Bøyum, 1968). Polymorphonuclear cells, neutrophils and eosinophils, were separated using the high density Ficoll method (Ferrante et al., 1982). In addition, eosinophils were purified by the method of Conesa et al. (1997). Purity of the different cell preparations was assessed by the lack of CD3/CD19/CD56 markers and the presence of CD16 (neutrophils), CD49d (eosinophils), and CD14 (monocytes) markers. For this purpose, cells were tagged with fluorescently labeled monoclonal antibodies: anti-CD16 PC5 (Becton Dickinson), anti-CD49d FITC (Beckman-Coulter), or anti-CD14 PerCP (BD Biosciences), then processed in a Q-Prep® workstation (Beckman-Coulter) and analyzed by flow cytometry according to Lara et al. (1997) in an Epics Elite cytometer (Beckman-Coulter) after previous alignment with DNA fluorescent check beads.

2.4. Oxidative burst assessment by flow cytometry

Peroxide production, as a measure of intracellular ROS, was determined by flow cytometry in a similar fashion as described earlier (Lara et al., 1997) with a minor modification. Briefly, after leukocyte purification, cells were loaded for 15 min at 37 °C with 1 μL of 20 mM 2′,7′-dichlorodihydrofluorescein diacetate, succinimidyl ester (OxyBURST® Green H_2DCFDA-SE, Invitrogen). H_2DCFDA-SE was used instead of dichlorofluorescein diacetate (DCFH-DA) as reported by Lara et al. (1997) due to the fact that

the succinimidyl ester is more specific than DCFH-DA according to the manufacturer. Cells (10^6/mL) were stimulated at 37 °C with 150 nM phorbol 12-myristate 13-acetate (PMA), scorpion venom or TzV chromatographic fractions and a time kinetic assay was performed. In the experiments where the effect of pretreatment with either PMA or TzV and subsequent stimulation with the same agents was explored, cells (10^6/mL) were incubated with the first agent for 15 min and then followed by a second, 15-min incubation with the second or previous stimulus. In all cases reactions were stopped by placing the tubes on ice. Subsequently, the cells were analyzed by flow cytometry and the fluorescence intensity evaluated (wavelength excitation, 488 nm; wavelength emission, 520 nm). Cell populations were gated using FALS *versus* 90° LS, as described previously (Lara et al., 1997); 5000 events were recorded for each point. Mean channel fluorescence intensity (which represents fluorescence intensity in logarithmic units) was reported as peroxide production. Cells were also labeled with anti-CD14 (monocytes), anti-CD16 (neutrophils) and anti-CD49d (eosinophils) fluorescently labeled monoclonal antibodies in order to ascertain specific peroxide production in each cell population.

The specificity of the assay was confirmed by incubating the cells with different concentrations of mannose (1, 5, 10 and 20 mM) or maleimide (0.5, 1, 2 mM) prior to the kinetic studies. Mannose (Rest et al., 1988) and maleimide (Deutsch, 1978) are specific inhibitors of the oxygen burst since they inhibit the hexose monophosphate shunt, which regenerates the NADPH substrate.

In addition, cells were incubated with staurosporine (Invitrogen) at 0.5 nM final concentration 10 min prior to the addition of the fluorochrome and performance of the kinetic assay in the case of the effect of crude venoms on neutrophils, monocytes, and eosinophils. Staurosporine was able to inhibit 90% of the PMA-mediated peroxide production (results not shown).

Cytochalasin B, a cytoskeleton disrupting compound known to augment the superoxide production elicited in human neutrophils by various stimuli (Wenzel-Seifert et al., 1997) was also used. Neutrophils were then treated with cytochalasin B (1 nM, Calbiochem) for 2 h prior to incubation with scorpion venom and measurement of ROS production.

2.5. Propidium iodide staining

To evaluate cellular necrosis after incubation with scorpion venom, neutrophils were resuspended in propidium iodide (PI)/Triton X-100 staining solution (0.1% (v/v) Triton X-100 (Sigma), 0.5 mg/mL DNAse-free RNAse A (Sigma) and 2 μg/ml PI (Roche) in phosphate buffer saline), incubated at 37 °C for 15 min and samples stored at 4 °C until analyzed (within 48 h) by flow cytometry (excitation at 488 nm and emission at 600 nm).

2.6. Cell binding assay

Scorpion venoms were labeled with fluorescein isothiocyanate (FITC) using the FluoReporter® FITC Protein Labeling Kit (Invitrogen) according to the manufacturer's instructions. The unbound dye was removed using Sephadex® G-10. No major protein loss was observed after binding and filtration. The cells were incubated with the labeled venom for 30 min at 37 °C and subsequently analyzed by flow cytometry (emission wavelength: 494 nm; excitation wavelength: 518 nm). Specific binding was assessed by subtracting the unspecific binding (calculated upon adding 10 μg of the unlabeled venom prior to the labeled venom). TzV fractions obtained by gel filtration chromatography (see Section 2.2) were labeled similarly as the whole venom. Binding and competition assays were performed and affinity constants (K) and EC_{50} values (defined as the protein concentration needed to achieve 50% of the maximum fluorescence value) were determined using the program GraphPad Prism, version 4 (GraphPad Software, San Diego, CA, USA).

2.7. Statistical analyses

Statistical significance of differences between treatments were determined using the repeated measures one-way analysis of variance implemented in the GraphPad Prism 4 software for Windows (version 4) (GraphPad Software, San Diego, CA, USA).

3. Results

3.1. Peroxide production elicited by TzV and TdV

Figs. 1 and 2 show the results of the evaluation of intracellular hydrogen peroxide production in human leukocytes exposed to TzV and TdV using H_2DCFDA-SE as a fluorescent substrate, which is oxidized mainly by hydrogen peroxide to green fluorescence (van Eeden et al., 1999). Three different cell populations were assessed, monocytes, neutrophils and eosinophils. Fluorescence at 0 μg/mL was subtracted from the data presented. TzV induced a significant increase ($p < 0.01$) with respect to TdV in intracellular peroxide production in neutrophils at 5 and 10 min of incubation

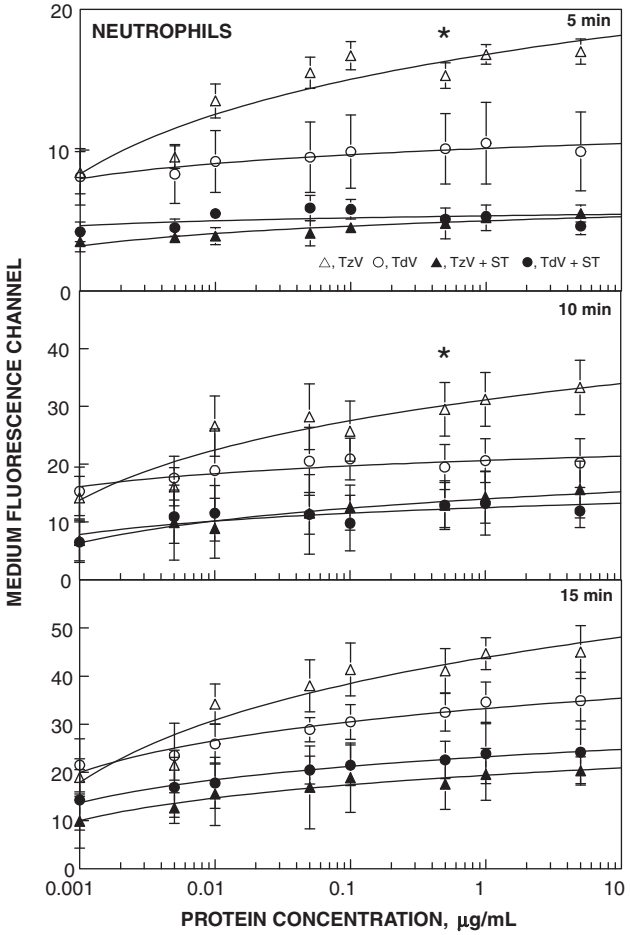

Fig. 1. Peroxide production by human neutrophils elicited by *Tityus* scorpion venoms. Fluorescence was recorded at 5, 10, and 15 min of incubation with either *T. zulianus* or *T. discrepans* venoms (0.001–5 μg/mL). The effect of pretreating the cells with 0.5 nM staurosporine (ST) is also shown. Values ($n = 6$) correspond to mean ± SEM. Significant differences (*, $p < 0.01$) were observed from 0.01 μg/mL venom concentration onwards between TzV- and TdV-stimulated peroxide production.

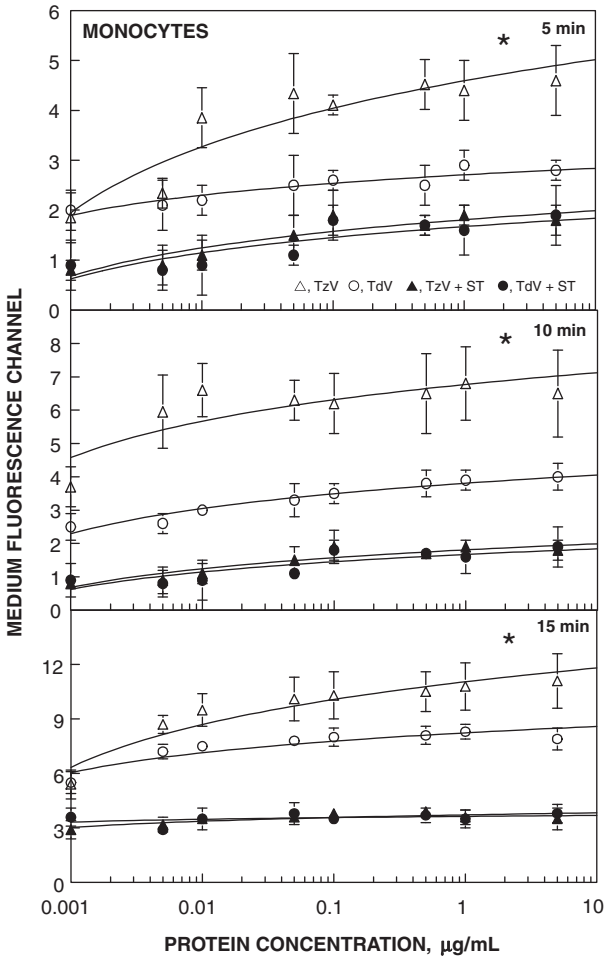

Fig. 2. Peroxide production by human monocytes elicited by *Tityus* scorpion venoms. Fluorescence was recorded at 5, 10, and 15 min of incubation with either *T. zulianus* or *T. discrepans* venoms (0.001–5 μg/mL). The effect of pretreating the cells with 0.5 nM staurosporine (ST) is shown. Values ($n = 6$) correspond to mean ± SEM. Significant differences (*, $p < 0.01$) were observed from 0.01 μg/mL venom concentration onwards between TzV- and TdV-stimulated peroxide production for 5 and 10 min of incubation. Significant differences between both venoms was observed in the case of 15 min-incubation for doses higher than 1 μg/mL.

and at doses higher than 0.01 μg/mL (Fig. 1). In the case of monocytes, TzV also induced a significant effect compared with TdV even at 15 min of incubation, albeit at doses higher than 1 μg/mL (Fig. 2). Although the ratio of fluorescence intensity in TzV-stimulated/non-stimulated cells was similar in neutrophils and monocytes, there were differences in the net increase in fluorescence between the two cell types. For instance, an elevation in medium fluorescence intensity from 8 to 20 was recorded for neutrophils compared to a 1.8–4.1 elevation for monocytes at 5 min of incubation for the same range of doses tested, representing a 5-fold increase in fluorescence in neutrophils compared to monocytes. Neither TdV nor TzV stimulated peroxide production in eosinophils at the doses tested on neutrophils and monocytes (data not shown). The stimulation of peroxide production by both TzV and TdV in neutrophils and monocytes was almost completely (>90%) abolished upon pre-incubation with 0.5 nM staurosporine, a known non-selective inhibitor of the protein kinase C-dependent peroxide production in human leukocytes (Lu et al., 1993). At TzV doses higher than 5 μg/mL, neutrophil necrosis was observed, determined by propidium iodide fluorescence incorporation (from 23.9 ± 4.9% at 20 min to 95% ± 4.9% at 60 min).

3.2. Spectral analyses of crude scorpion venoms and chromatographic fractions

Fig. 3 shows a comparison of the MALDI-TOF mass spectra of crude TzV and TdV. Both venoms contained peptides in the 6.5–7.5 and 2.5–4.0 kDa mass ranges, known to target ion channels in excitable cells (Possani and Rodríguez de la Vega, 2006). TzV contained low molecular mass components in the 1.5–2.5 kDa mass range which were lacking in the *T. discrepans* spectrum. To identify the *T. zulianus* components responsible for the stimulation of peroxide production in leukocytes, venom was fractionated using gel filtration chromatography in Sephadex® G-50 (Fig. 4, top left inset). Protein composition of crude TzV and fractions was analyzed by SDS–PAGE in 15% gels (Fig. 4, top right inset). There were no molecular mass components within the detection range of the MALDI-TOF technique (<14 kDa) for fraction TzI (data not shown) whereas TzII and TzIII harbored lower molecular mass components. Fig. 4 also shows a comparison of the MALDI-TOF spectra of TzV and chromatographic fractions TzII and TzIII. While TzII contains most low molecular mass components present in TzV, fraction TzIII contains lower amounts of the 6.5–7.5 kDa components and is particularly enriched in a peptide of mass 2087.6 Da, also present in the TzV spectrum.

3.3. Assessment of the activity of TzV chromatographic fractions on the stimulation of peroxide production

Peroxide production in human neutrophils and monocytes was tested as a result of incubation with fractions TzI, TzII, and TzIII (from 1 ng/mL up to 1 μg/mL) in comparison with crude TzV (Figs. 5 and 6). Peroxide production mediated by TzII and TzIII at the highest concentration used (1 μg/mL) was significantly higher ($p < 0.01$) than the effect of fraction TzI on both neutrophils (for 5 and 10 min of incubation) and monocytes (for 5, 10, and 15 min of incubation). Activity of either TzII or TzIII was not significantly different from TzV-mediated stimulation of neutrophils and monocytes. Neither TzII nor TzIII stimulatory action matched stimulation by the crude venom in both cell types. There were differences in the net increase in fluorescence between neutrophils and monocytes stimulated with TzII and TzIII for 5 and 10 min of incubation. For instance, the average fluorescence intensity in TzII-stimulated neutrophils increased from 8 to 18 at 5 min of incubation whereas in monocytes it increased from 1.8 to 3 for the same range of doses tested, representing an 8-fold fluorescence increase in neutrophils compared to monocytes. For the 10-min incubation, increase in fluorescence intensity was 3.5-fold in neutrophils with respect to monocytes.

3.4. Cell binding

Fig. 7 shows the results of binding of FITC-labeled TzV and fractions TzI, TzII, and TzIII to human neutrophils, monocytes, and eosinophils. The cell binding assays recorded specific venom binding to leukocyte membranes since fluorescence emission was quenched by trypan blue (from 5.5 to 0.4 medium channel fluorescence intensity). No marked increase in intracellular fluorescence was observed after incubating cells up to 60 min. Binding of TzV and fractions derived thereof to neutrophils and monocytes was significantly higher ($p < 0.05$) compared to eosinophils. No significant difference was found in the binding to neutrophils and monocytes. Affinity constants (K) were determined using FITC-labeled fractions TzI, TzII, and TzIII. K values are very similar for all fractions regardless of the cell type used (0.03–0.05 μg). EC_{50} values (in neutrophils) were not significantly different between fractions TzIII (0.02 μg/mL), TzII (0.05 μg/mL) and TzI (0.1 μg/mL). Treatment of neutrophils with cytochalasin B 2 h prior to incubation

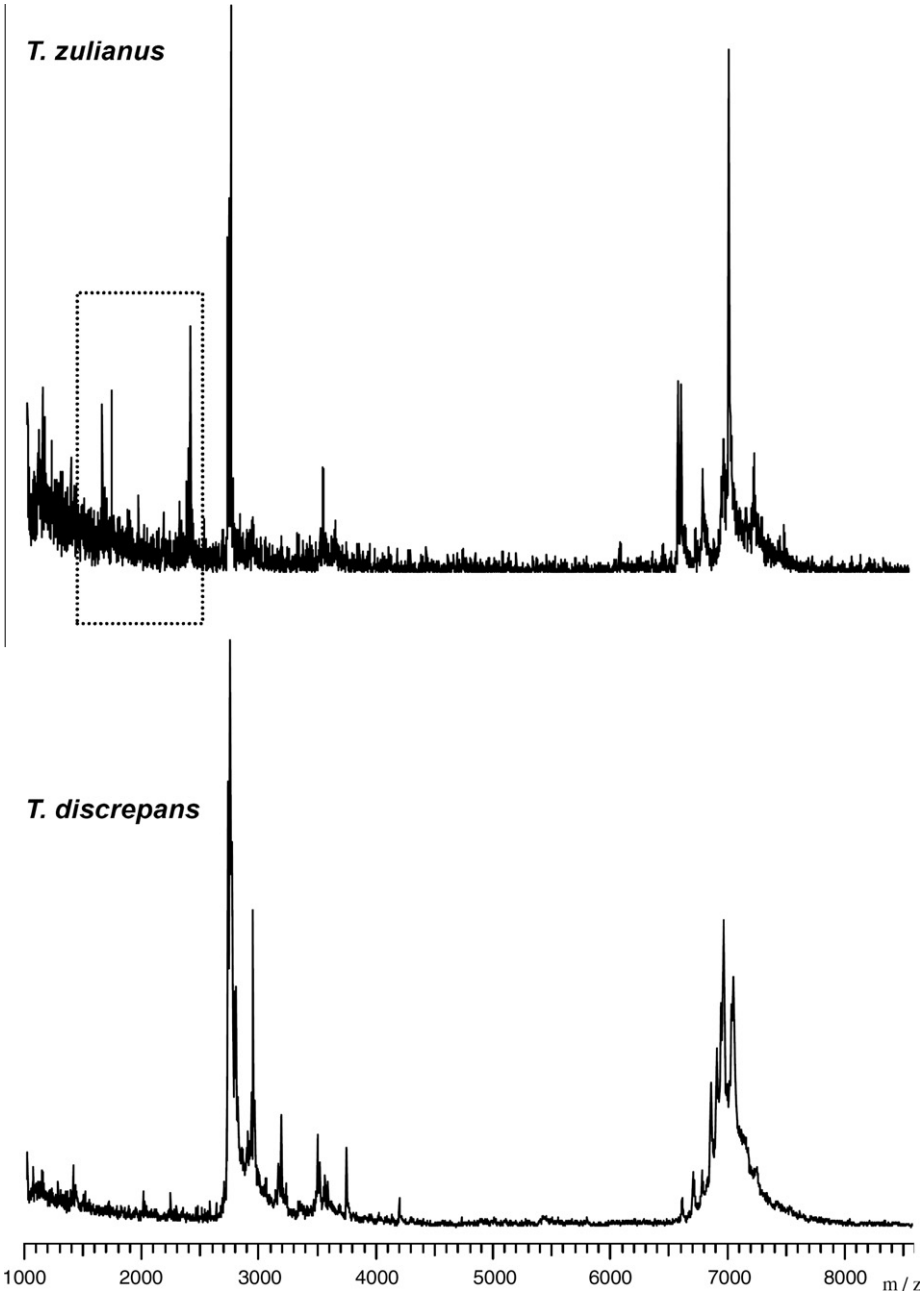

Fig. 3. Composition of *Tityus zulianus* and *Tityus discrepans* scorpion venoms determined by MALDI-TOF mass spectrometry. Selected section in the TzV spectrum corresponds to peptides in the 1.5–2.5 kDa mass range. Spectra are representative of three independent analyses performed with different scorpion venom pools.

with TzV produced no significant difference in peroxide production between treated and non-treated cells (data not shown).

3.5. Combined effect of PMA and TzV on human neutrophils

In order to ascertain the mechanism by which the venom induces an increase in peroxide production, the cells were treated with 150 nM PMA 15 min before or after being primed with TzV. Table 1 shows that pretreatment of neutrophils with 150 nM PMA resulted in a significantly enhanced response ($p < 0.01$, $n = 4$) to a second stimulus with TzV (5 μg/mL) while pretreatment of cells with TzV produced no response to a subsequent addition of PMA. There was no significant difference in peroxide production between cells stimulated with TzV prior or subsequent to PMA stimulation.

4. Discussion

Previous reports have documented the effect of the Brazilian scorpion *T. serrulatus* venom and Na_v-active toxins derived thereof on murine macrophages (Petricevich, 2002; Petricevich et al., 2007, 2008; Petricevich and Lebrun, 2005; Petricevich and Peña, 2002), describing its stimulation of pro-inflammatory cytokine production and phagocytic activity. The data presented in this work are the first to show that components capable of directly eliciting peroxide production in human neutrophils, as a measure of intracellular reactive oxygen species (ROS), are produced by *Tityus* scorpions. Earlier studies have recognized the fact that venoms from *T. zulianus* and *T. discrepans*, two of the most medically important scorpion species in Venezuela, differ functionally and antigenically

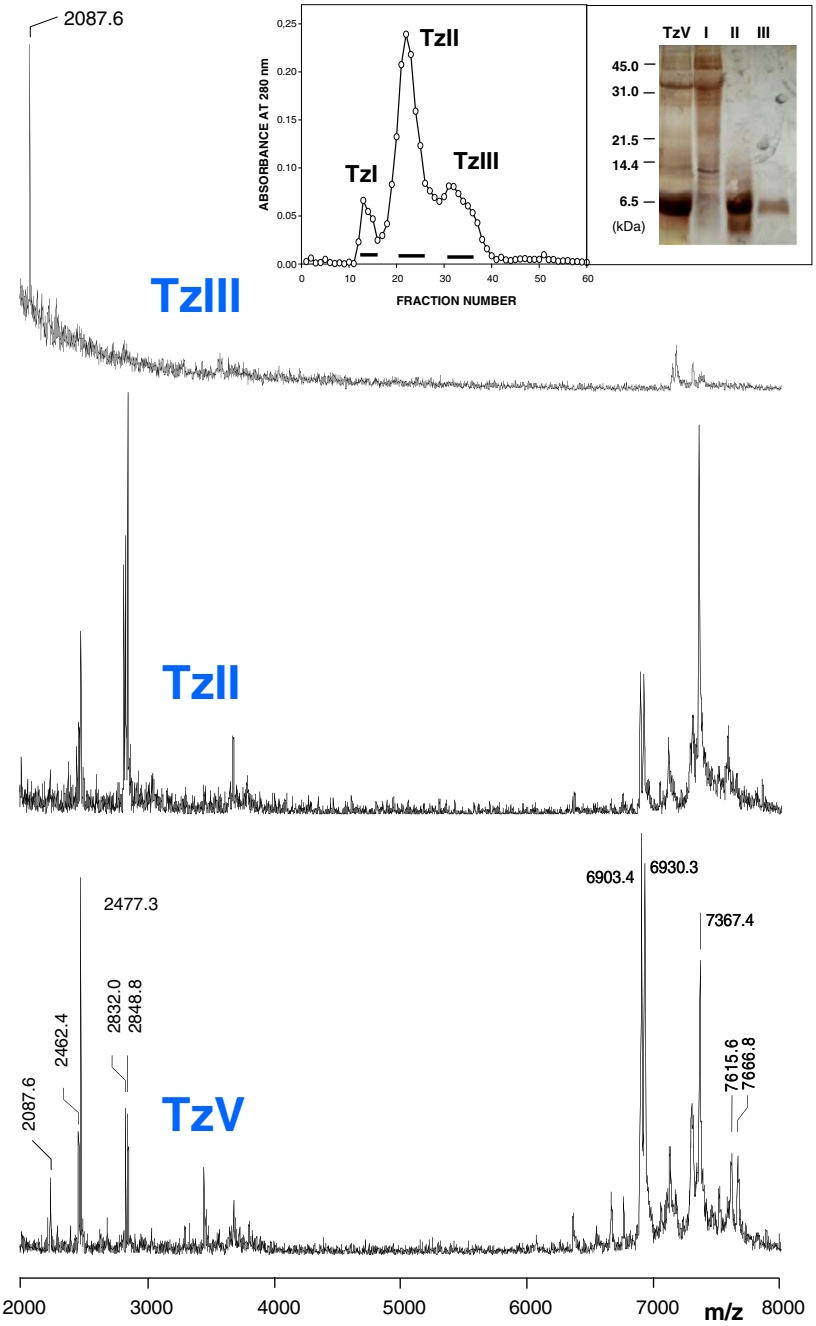

Fig. 4. Mass spectral analyses of TzV chromatographic fractions. (Panels from top to bottom) TzIII, TzII and TzV: MALDI-TOF spectra of chromatographic fractions and crude TzV, respectively. All spectra are representative of at least three analyses of fractions obtained by chromatography of independent venom samples. Top left inset: elution profile from Sephadex G-50 columns of TzV (5 mg protein) recorded at 280 nm, indicating fractions TzI, TzII and TzIII subjected to mass spectrometry and used in peroxide production experiments. Top right inset: silver-stained SDS–PAGE gel (15%) showing composition of fractions TzI, TzII, TzIII and crude TzV (15 μg per lane).

(Borges et al., 2004, 2008). This work reinforces these studies as our results show that the peroxide production stimulated by TzV on human neutrophils (and monocytes to a lesser extent) is significantly more potent than that mediated by TdV, implying that *Tityus* species are diverse in terms of the immunomodulatory action of their venom components. TzV, as opposed to TdV, specifically promotes peroxide production in neutrophils and induces necrosis in these cells at concentrations higher than 5 μg/mL. *T. zulianus* either synthesizes species-specific, ROS-stimulating products which are very active or, alternatively, it contains components similar to those produced by *T. discrepans* at a higher concentration. Significantly, we show that the mass spectral fingerprint of TzV contains higher abundance of peptides in the 1.5–2.5 kDa mass region (Fig. 3), characteristic of the non-disulfide-bridge family of scorpion peptides possessing cytolytic and antimicrobial activity (Zeng et al., 2005).

Several pathways could be the target for the ROS stimulatory activity of TzV in neutrophils at sub-necrotic concentrations. The targeted cascade should be PKC-dependent as the TzV effect is suppressed by staurosporine through its PKC inhibition, which entails binding at a conserved site in the catalytic domain of broad variety of kinase isotypes (Ward and O'Brian, 1992). ROS production pathways in granulocytes involve NADPH oxidase (NOX)-dependent and independent cascades but the main source in neutrophils are

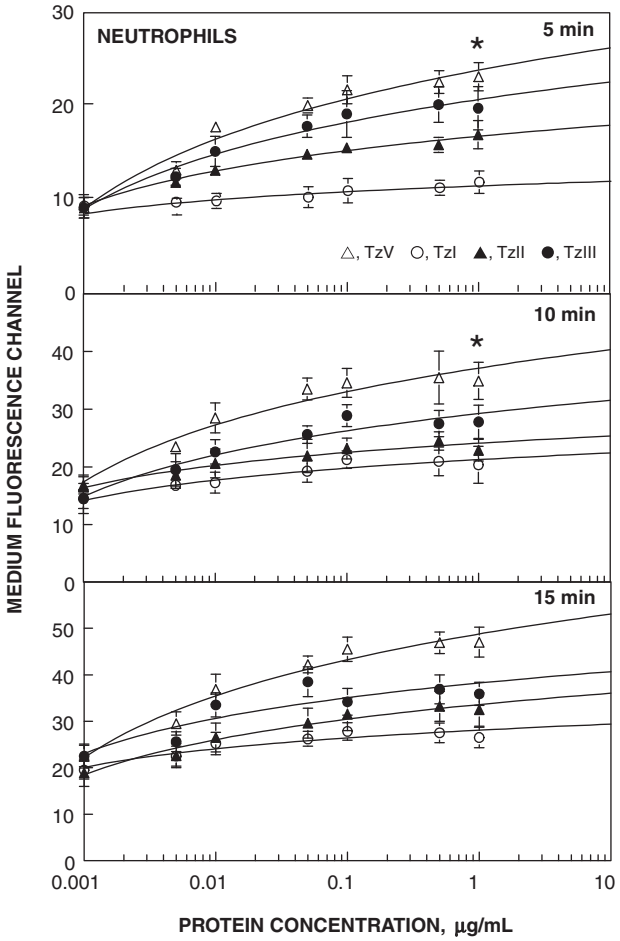

Fig. 5. Peroxide production by human neutrophils elicited by TzV and TzI, TzII, and TzIII chromatographic fractions derived thereof. Fluorescence was recorded at 5, 10, and 15 min of incubation with either TzV or its fractions (0.001–1 µg/mL). Values ($n = 6$) correspond to mean ± SEM. Fluorescence at 0 µg/mL was subtracted from the data presented. A significant difference (*, $p < 0.01$) was observed at 1 µg/mL between TzI- and TzIII-stimulated peroxide production at the indicated time points.

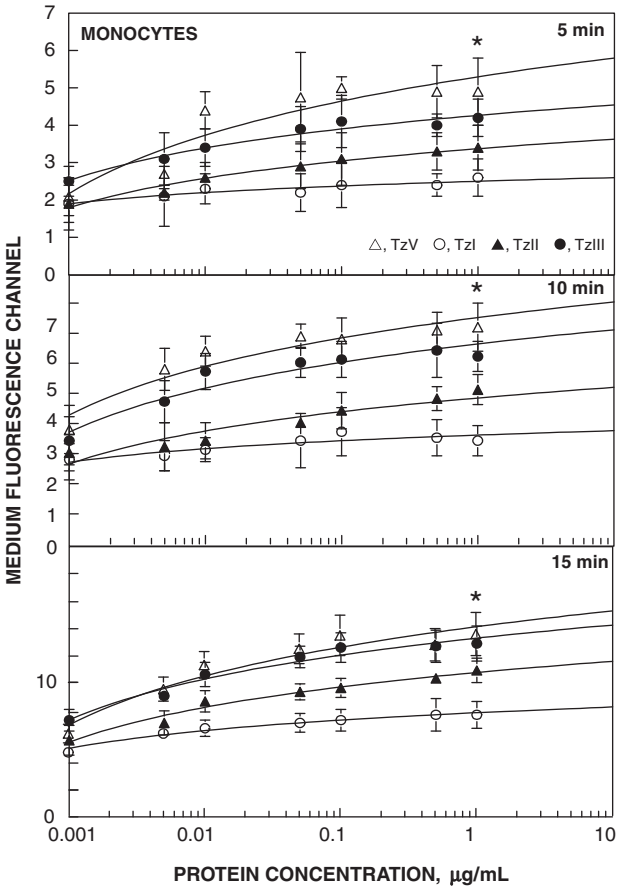

Fig. 6. Peroxide production by human monocytes elicited by TzV and TzI, TzII, and TzIII chromatographic fractions derived thereof. Fluorescence was recorded at 5, 10, and 15 min of incubation with either TzV or its fractions (0.001–1 µg/mL). Values ($n = 6$) correspond to mean ± SEM. A significant difference (*, $p < 0.01$) was observed at 1 µg/mL between TzI- and TzIII-stimulated peroxide production at the indicated time points.

plasma membrane-bound and granule and phagosomal NOXs since NOX-independent ROS production mainly involves mitochondrial oxidative phosphorylation. Neutrophil mitochondria are low in number and appear to perform little, if any, oxidative phosphorylation (Maianski et al., 2004). PKC-dependent phosphorylation is crucial for NOX activation, which is the primary source of superoxide anion in neutrophils and, in turn, after dismutation by superoxide dismutase, of hydrogen peroxide (Sheppard et al., 2005). ROS production through oxidase activation requires assembly of the cytosolic components p47*phox*, p67*phox*, and rac2 and interaction with cytochrome b_{558}. Such an assembly of the active NOX for ROS generation requires phosphorylation and translocation of the cytosolic factor p47*phox* which is phosphorylated by several PKC isoforms (Vignais, 2002).

The cell-type specificity of the TzV effect can throw light on the identity of the ROS production pathway being targeted by the venom. TzV-elicited ROS production (at 5 µg/mL, 5 min of incubation) was 5-fold higher in neutrophils with respect to monocytes and negligible in eosinophils. After labeling TzV and chromatographic fractions with FITC, only marginal protein binding to eosinophils was found compared to neutrophils and monocytes. The fact that affinity constants for FITC-labeled fractions were similar regardless of the cell type suggests that differences in TzV-mediated ROS stimulation between neutrophils and eosinophils are related to signaling components beyond the venom primary (possibly membrane-bound) target. The differential response of eosinophils and neutrophils to TzV may be attributable, at least in part, to the fact that neutrophils preferentially generate ROS inside cells (Lacy et al., 2003) and that our detection system measures mainly intracellular peroxide using the fluorescent substrate H_2DCFDA-SE. Although both neutrophils and eosinophils are granulocytes and use the same NOX components to generate superoxide, the amount, location and regulation of the oxidase is different in these cell types (Kato et al., 2005; Someya et al., 1997). Eosinophils express more NOX than neutrophils in humans and the majority of the oxidase is located on the plasma membrane (Lacy et al., 2003; Someya et al., 1997). By contrast, the vast majority of neutrophil NOX is located intracellularly, i.e. in granule or phagosomal membranes, which is regulated differently to plasma membrane NOX (Granfeldt et al., 2002). In fact, neutrophils express higher levels, compared to eosinophils, of methionine–sulfoxide–reductase enzymes in order to cope with the potential damage elicited by intracellular ROS (Achilli et al., 2008). This difference in NOX location is consistent with the distinct functional roles of neutrophils and eosinophils. Neutrophils kill primarily by phagocytosing their targets, whereas eosinophils kill nonphagocytosable targets by secreting toxic agents, including ROS (Abraham, 2003; Hogan et al., 2008). The reduced response of monocytes to TzV can be explained by differences in the NOX activation pathways between neutrophils and these cells. Agents that activate NOX in neutrophils do not necessarily trigger NOX in monocytes/

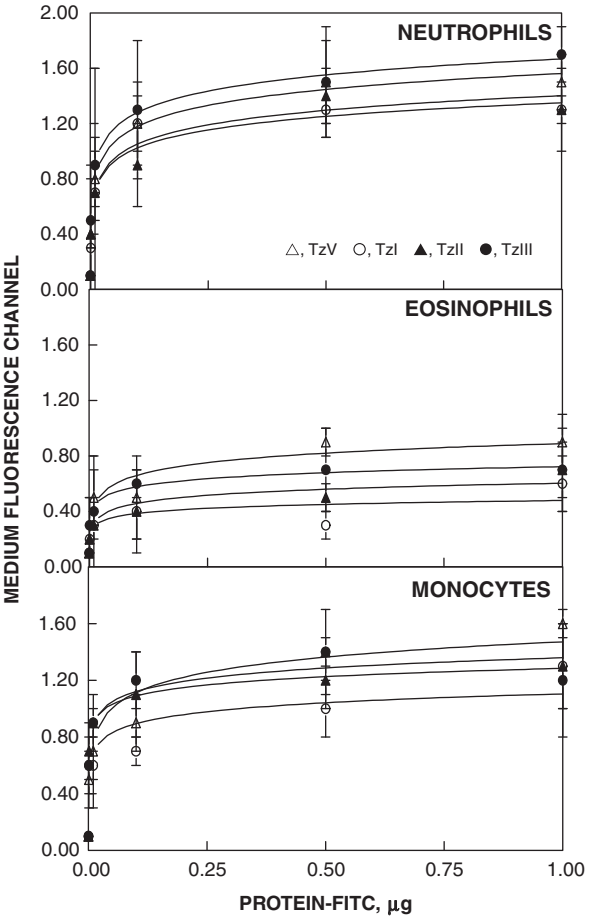

Fig. 7. Binding of crude TzV and TzI, TzII, and TzIII chromatographic fractions derived thereof to human neutrophils, eosinophils and monocytes. Venom proteins were labeled with FITC as described in Section 2. Values ($n = 6$) correspond to mean ± SEM.

Table 1
Effect of priming and subsequent stimulation by TzV or PMA on the peroxide production of human neutrophils.

Priming stimulus[a]	Second stimulus	Peroxide production[b] (Fluorescence units)
PMA	None	31 ± 2.6
	TzV	48 ± 2.6*
TzV	None	45.0 ± 5.0
	PMA	43.3 ± 2.1

[a] Neutrophils (10^6/mL) were exposed to the indicated stimuli at 37 °C for 15 min before the addition of a second stimulus. After a further incubation for 15 min, reactions were terminated and fluorescence assessed by cytometry as described in Section 2. Concentrations of stimuli for priming and second stimulation were: PMA, 150 nM; TzV, 5 µg/mL.

[b] Values ($n = 4$) are mean ± SEM.

* $p < 0.01$ with respect to the experiment with PMA as priming stimulus without second stimulation).

macrophages (Cathcart, 2004). For instance, monocytes preferentially express Rac1 as the intracellular regulatory GTPase for NOX activation (Zhao et al., 2003) and Rac1 binds with a 6-fold less affinity with p67*phox* than Rac2 (Dorseuil et al., 1996).

Phorbol esters, such as PMA, directly activate PKC mimicking the effects of the natural PKC activator, diacylglycerol (Castagna et al., 1982), and have been suggested to activate NOX through redistribution of PKC and phosphorylation of several proteins including p47*phox* (Karlsson et al., 2000). Given the possibility that TzV mediates intracellular ROS generation through a neutrophil-specific, PKC-dependent signaling cascade, and considering that PMA has been used as a model agonist to investigate intracellular NOX activity in neutrophils (Karlsson and Dahlgren, 2002; Karlsson et al., 2000), we investigated whether the effects of TzV and PMA on peroxide production could be influenced by pretreatment with either agent. It is generally found that prior exposure of neutrophils to one stimulus could potentiate the respiratory burst to the subsequent addition of a second heterologous stimulus (McPhail et al., 1984). Pretreatment of neutrophils with PMA produced a modest, although significant stimulation of peroxide production upon subsequent exposure to TzV, suggesting that the venom and PMA probably engage neutrophil activation through overlapping signaling cascades. One possible explanation for the TzV-mediated activation of neutrophils is that the venom could increase the intracellular [Ca^{+2}] in a mechanism reminiscent of the activity of A23187 and other calcium ionophores, which potentiate ROS production elicited by phorbol esters (Brown and Ganey, 1995; Dahlgren et al., 1992; French et al., 1987; McPhail et al., 1984). In this sense, pore-forming peptides promoting Ca^{+2} influx have been described in the venoms of *Parabuthus* and *Opistophthalmus* scorpions (Moerman et al., 2002).

Another explanation is that venom components directly activate PKC isoforms involved in intracellular NOX-mediated ROS production, which is known to involve different kinase isoforms compared to the membrane-bound NOX (Karlsson et al., 2000). The peptide parabutoporin, from the venom of the scorpion *Parabuthus schlechteri*, potently abolishes ROS production triggered by PMA in neutrophils (Willems et al., 2002), due to inhibition of PKC-driven phosphorylation of p47*phox* and its subsequent membrane translocation (Remijsen et al., 2006). As opposed to parabutoporin, our evidence indicates the presence of peptides in TzV that are direct or indirect (i.e. through an increased intracellular [Ca^{+2}]) PKC activators. The contribution of PKC activation to the TzV-induced respiratory burst may also explain the reduced response to a subsequent addition of PMA (after TzV stimulation) which could be limited by the availability of additional activatable PKC for PMA binding (McPhail et al., 1984). The fact that the TzV-mediated activation is insensitive to cytochalasin B suggests that actin polymerization does not play a role in the effect as seen in the case of chemotactic factors, plant lectins, and ionophores (Wenzel-Seifert et al., 1997).

Our data also shows that TzV components responsible for the stimulatory effect are low molecular mass components, contained in gel filtration fractions TzII and TzIII. TzV stimulatory components appear to act synergistically in their effect on neutrophils because activity of TzII or TzIII did not match stimulation of crude venom (Fig. 5). TzII is mainly composed of short- (3–4 kDa) and long-chain (6–7 kDa) peptides, known to modify potassium and Na_v channels in excitable cells, respectively (Possani and Rodríguez de la Vega, 2006), whereas TzIII is enriched in a 2087.6 Da peptide and lower amounts of the 6–7 kDa long-chain peptides eluting in TzII (Fig. 4). Further fractionation of TzII and TzIII is warranted to assign the peroxide stimulating activity to either group of TzV peptides.

In summary, our evidence suggests the existence of newly identified scorpion peptides produced by *T. zulianus* that activate generation of intracellular ROS in human neutrophils through a PKC-sensitive mechanism. Given the cell-type specificity of the TzV effect, the ROS-stimulating TzV components may have a role to play in the *T. zulianus* envenomation syndrome, which entails more severe cardiorespiratory complications than those seen in the case of TdV (Borges et al., 2004; Mazzei de Dávila et al., 2002). It has been suggested that the pronounced PAF-driven neutrophilia detected in pulmonary tissues after envenomation by *T. serrulatus* contributes to the venom-mediated lung injury in children (Borges et al., 2000). Neutrophil activation is strongly related to organ dysfunction that occurs during systemic inflammation

and, in different models of acute lung injury, neutrophils that infiltrate the lungs and migrate into the airways contribute to oxidant-induced cellular and tissue damage (Abraham, 2003; Moraes et al., 2006). It should also be noted that increased intracellular ROS concentrations are pro-inflammatory in neutrophils, acting through a p38 MAP kinase-dependent mechanism that results in enhanced nuclear accumulation of NF-κB and increased expression of NF-κB-dependent pro-inflammatory cytokines (Moraes et al., 2006; Mitra and Abraham, 2006). Our findings should stimulate further research on the role played by neutrophils in the physiopathology of the human envenomation by *T. zulianus* and possibly by other species within this scorpion genus.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Acknowledgments

We acknowledge the help of Mrs. Eglys Chirinos during scorpion maintenance and venom collection at the Instituto de Medicina Experimental, Central University of Venezuela. Financial support was from Grants FONACIT 2001000672 (to A.B.) and FONACIT G2005000389 (to J.B.D).

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