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Phospholipase-D activity and inflammatory response induced by brown spider dermonecrotic toxin: Endothelial cell membrane phospholipids as targets for toxicity

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ABSTRACT

Brown spider dermonecrotic toxins (phospholipases-D) are the most well-characterized biochemical constituents of *Loxosceles* spp. venom. Recombinant forms are capable of reproducing most cutaneous and systemic manifestations such as dermonecrotic lesions, hematological disorders, and renal failure. There is currently no direct confirmation for a relationship between dermonecrosis and inflammation induced by dermonecrotic toxins and their enzymatic activity. We modified a toxin isoform by site-directed mutagenesis to determine if phospholipase-D activity is directly related to these biological effects. The mutated toxin contains an alanine substitution for a histidine residue at position 12 (in the conserved catalytic domain of *Loxosceles intermedia* Recombinant Dermonecrotic Toxin — LiRecDT1). LiRecDT1H12A sphingomyelinase activity was drastically reduced, despite the fact that circular dichroism analysis demonstrated similar spectra for both toxin isoforms, confirming that the mutation did not change general secondary structures of the molecule or its stability. Antisera against whole venom and LiRecDT1 showed cross-reactivity to both recombinant toxins by ELISA and immunoblotting. Dermonecrosis was abolished by the mutation, and rabbit skin revealed a decreased inflammatory response to LiRecDT1H12A compared to LiRecDT1. Residual phospholipase activity was observed with increasing concentrations of LiRecDT1H12A by dermonecrosis and fluorometric measurement *in vitro*. Lipid arrays showed that the mutated toxin has an affinity for the same lipids LiRecDT1, and both toxins were detected on RAEC cell surfaces. Data from *in vitro* choline release and HPTLC analyses of LiRecDT1-treated purified phospholipids and RAEC membrane detergent-extracts corroborate with the morphological changes. These data suggest a phospholipase-D dependent mechanism of toxicity, which has no substrate specificity and thus utilizes a broad range of bioactive lipids.

1. Introduction

Dermonecrotic toxins are biologically and biochemically well-characterized constituents of the Brown spider (genus *Loxosceles*) venom complex mixture. Most of the described toxic effects associated with brown spiders, such as an intense inflammatory response at the bite site, hemostasis disorders, renal injury, platelet aggregation, and the typical necrotic lesion of the skin, can be experimentally reproduced using dermonecrotic toxins [1–5].

Histological findings for animals exposed to whole venom have shown subendothelial blebs, vacuoles, endothelial cell membrane degeneration of the blood vessel walls, and fibrin and thrombus formation [6,7]. Moreover, Paludo et al. [8] demonstrated morphological alterations in an endothelial cell line derived from the rabbit aorta (RAEC) treated with whole venom such as cell retraction, homophilic disadhesion, and an increase in filopodia projections. In addition, venom was observed to bind to the cell surface and extracellular matrix. These direct cytotoxic effects on blood vessel cells could be responsible for triggering intense infiltration of inflammatory cells. In addition, they could indirectly lead to leukocyte and platelet activation, disseminated intravascular coagulation, and an increase in vessel permeability.

Dermonecrotic toxins are very characteristic and conserved molecules among *Loxosceles* species. Currently, more than ten complete mRNA sequences for *Loxosceles* spp. dermonecrotic toxins have been deposited in the nucleotide databases [3,9]. These toxins were biochemically reported as sphingomyelinase-D (or sphingomyelin phosphodiesterase D; E.C. 3.1.4.41) family members due to their

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ability to catalyze the hydrolysis of sphingomyelin [1,2]. Recently, Lee and Lynch [10] suggested that the dermonecrotic toxin family should be more accurately named phospholipases-D due to their additional hydrolytic activity upon glycerophospholipids.

Six isoforms of phospholipase-D were cloned from a cDNA library of *L. intermedia* gland venom and then expressed; they were shown to have similar toxic effects to those of native venom toxins [11–15]. Appel et al. [11] performed an alignment analysis of the cDNA-deduced amino acid sequences for LiRecDT1 (ABA62021), LiRecDT2 (ABB69098), LiRecDT3 (ABB71184), LiRecDT4 (ABD91846), LiRecDT5 (ABD91847), and LiRecDT6 (ABO87656). The results of this alignment corroborated the crystal structure analysis of a dermonecrotic toxin described by Murakami et al. [16] that suggested conserved residues at the proposed catalytic site.

Two histidine residues (H12 and H47) are postulated to be responsible for an acid–base reaction stabilized by Mg^{+2} ion coordination at the catalytic site. In the intermediate steps of the hypothetical mechanism, the His12 residue must be deprotonated for the subsequent nucleophilic attack of a water molecule to succeed. This mechanism can be followed by the final release of ceramide 1-phosphate (C1P) from the sphingomyelin substrate or lysophosphatidic acid (LPA) from a lysophospholipid [16,17].

Studies on lipid metabolism have shown that glycerophospholipids and sphingolipids are not simply structural constituents of cell membranes. Bioactive lipid mediators have been shown to play a major role in complex signaling pathways that control several cellular dynamics such as cell growth, survival, differentiation, and motility. They have also been shown to play roles in various pathophysiologic processes, all of which involve several G-protein-coupled receptors (GPCRs) and kinase cascades [18–20].

Pettus et al. [21] showed that C1P, rather than ceramide, functions as the proximal mediator of arachidonic acid release. Exogenous C1P alone can stimulate arachidonic acid release and prostaglandin E2 formation. Treatment of cells with spider venom sphingomyelinase-D to produce C1P from membrane sphingomyelin can elicit the arachidonic acid response [22]. Likewise, phospholipase-D can induce LPA formation by catalyzing the hydrolysis of a *lyso*-phospholipid precursor. In quiescent fibroblasts, exogenous PLD (from *Streptomyces chromofuscus*) generated bioactive LPA from pre-existing lysophosphatidylcholine in the outer membrane leaflet, resulting in the activation of G-protein-coupled LPA receptors and subsequent activation of the Ras, Rho, and Ca^{2+} signaling pathways [23]. Similarly, recombinant SMaseD from *L. laeta* was able to hydrolyze lysophosphatidylcholine to produce LPA and choline. Recombinant PLD from *L. recluse* also degraded several purified lysophospholipids and sphingomyelin substrates [10,24]. Gene expression profiles of human fibroblasts treated with *L. recluse* isoform I recombinant toxin (SMD) showed the upregulation of genes related to human cytokines and genes involved in the glycosphingolipid metabolism pathway. Furthermore, they speculated that sphingomyelinase activity on cell membranes, which results in ceramide 1-phosphate (C1P) production, induces the pro-inflammatory molecules NF-kB and IL-8 [25].

In the literature, there are several proteins with enzymatic activities (as well as catalytic domain structures) that are not responsible for the toxicity observed in experimental systems. Snake venom phospholipases A2 (PLA2) present remarkable diversity in their biological effects in addition to their catalytic activity. Sequence alignment analyses of functionally related PLA2 are frequently used to predict the structural determinants of these effects, and the predictions are subsequently evaluated by site-directed mutagenesis experiments and functional assays [26]. In this way, Sakamoto et al. [27] demonstrated that modified CyaAs (calmodulin-dependent adenylate cyclases from *Bordetella pertussis*) display hemolytic activity identical to the original toxin.

The absence of a clear correlation between catalysis and pharmacological activity and the diversity of biological effects raises questions regarding the structural basis of these biological functions. Nephrotoxicity and hemolysis directly induced by phospholipase-D activity were already reported in literature [10,28,29]. However, dermonecrosis *in vivo* and inflammatory response triggered by brown spider phospholipase-D toxin were not yet described as directly dependent on its catalytic activity. In this report, we used a site-directed mutagenesis strategy to clone and express a mutated form of the previously reported dermonecrotic toxin known as LiRecDT1 (ABA62021) [12]. The substitution of a histidine residue at position 12 in the amino acid sequence with an alanine was performed to modify the organization of the active site and interfere with the catalytic reaction of the enzyme.

We investigate the correlation between the toxicity reported in loxoscelism (mainly characterized by dermonecrosis and inflammatory disturbance) and the biochemical and catalytic mechanism of phospholipase-D. We report evidence demonstrating the dependence of the biological effects on phospholipase-D activity of dermonecrotic toxin *in vivo* and *in vitro*, bringing new insights to the molecular mechanism of phospholipase-D activity.

2. Materials and methods

2.1. Reagents

Whole venom from *L. intermedia* obtained by electrostimulation (15 V) of the cephalothorax of spiders captured in the wild was solubilized in PBS and maintained frozen until used, as described by Feitosa et al. [30]. Polyclonal antibodies to *L. intermedia* phospholipase-D toxin and whole venom were produced in rabbits as described by Chaim et al. [12]. Hyperimmune IgGs were purified from sera using a mixture of Protein-A and Protein-G Sepharose beads (GE Healthcare, Chalfont St Giles, England), as recommended by the manufacturer. Fluorescein-conjugated anti-rabbit IgG and alkaline phosphatase-conjugated or peroxidase-conjugated anti-rabbit IgG were purchased from Sigma (St. Louis, USA).

2.2. Site-directed mutagenesis of LiRecDT1

Cloning from the venom gland cDNA library was performed following the methods of Chaim et al. [12]. GenBank data deposition information for *L. intermedia* cloned cDNA can be found under LiRecDT1 (DQ218155). The cDNA corresponding to the mature phospholipase-D original protein was amplified by PCR. The sense primer used was 30Rec (5′-CTCGAGGCAGGTAATCGTCGGCCTATA-3′), which was designed to contain a XhoI restriction site (underlined) plus the sequence for the first seven amino acids of the mature protein. The antisense primer used was 30Rec (5′-CGGGATCC**TA**TTTCTTGAATGTCACCCA-3′), which contains a BamHI restriction site (underlined) and a stop codon (bold). The PCR product was cloned into the pGEM-T vector (Promega, Madison, USA), subcloned into pET-14b (Novagen, Madison, USA) and digested with XhoI and BamHI. The mutated toxin was obtained using the Megaprimer PCR method adapted from Sambrook and Russell [31]. This protocol was performed using three rounds of PCR to carry out site-directed mutagenesis in the LiRecDT1 sequence, and the resulting mutated toxin was named LiRecDT1 H12A. Briefly, the first round of PCR included site-directed mutagenesis of the first histidine amino acid residue using the forward primer T7, which anneals to the pSPORT vector 5′ portion, and the special reverse primer P1H12A (5′-ATTTACCATG**GC**CCCCATGATC-3′), which contains the codon substitute for alanine plus the sequence for the other original amino acids. The "megaprimer" for the next round was obtained by agarose gel electrophoresis of the first PCR product (~200 bp) and purified using the QIAquick Gel Extraction Kit (QIAGEN, Hilden, Germany). In the second step, PCR was performed to obtain the mutated product using the "megaprimer" as the sense primer and 30Rec as the antisense

primer (3′ BamHI restriction site). The second PCR product was subjected to the same protocol of agarose gel purification. In the final step of mutagenesis, a third PCR reaction was performed to insert the 5′ XhoI restriction site from the second PCR product by using 30Rec as the sense primer and 30Rec as the antisense primer. Finally, this last PCR product was digested with restriction enzymes and purified by agarose gel electrophoresis. The mutated product was then cloned into the pGEM-T vector (Promega) and subcloned into pET-14b (Novagen). The correct construct was confirmed by miniprep sequencing.

2.3. Original (LiRecDT1) and mutated LiRecDT1 (LiRecDT1 H12A) recombinant toxin expression and purification

Both recombinant constructs were expressed as fusion proteins with a poly-(6X) His tag at the N-terminus as previously described by Chaim et al. [12]. Briefly, pET-14b/*L. intermedia* cDNA constructs were transformed into One Shot *E. coli* BL21(DE3)pLysS competent cells (Invitrogen). Expression conditions were standardized for induction with IPTG (isopropyl β-D-thiogalactoside) for 3.5 h at 30 °C in 1 L of LB broth cultures. Cell suspensions were sonicated with six 10-second cycles at low intensity, and lysed materials were centrifuged (20,000×*g*, 20 min). The recovered supernatants were incubated for affinity chromatography with 1 ml of Ni^{2+}-NTA agarose beads for 1 h at 4 °C. Recombinant protein/beads suspensions were loaded into a column and exhaustively washed until the OD at 280 nm reached 0.01 (washing buffer: 50 mM sodium phosphate pH 8.0, 500 mM NaCl, 20 mM imidazole). Recombinant proteins were eluted with 10 ml of elution buffer (washing buffer, but 250 mM imidazole). Protein expression analysis on 12.5% SDS-PAGE was performed for purity and was stained with Coomassie Blue dye. For experimental negative controls, a recombinant toxin with similar molecular mass and obtained from the same cDNA library was used. This control toxin was characterized as an "astacin-like metalloprotease" [32] and caused no dermonecrosis, as evidenced by the dermonecrotic assay following injection into rabbit skin (data not shown).

2.4. Circular dichroism spectra measurements

Recombinant original and mutated toxins were dialyzed at 4 °C against 10 mM sodium phosphate buffer, pH 7.4, and their spectra were recorded in a Jasco J-810 spectropolarimeter (Jasco Corporation, Tokyo, Japan) using a 1-mm gap cuvette. Each reported spectrum (0.5 nm interval) is the average of eight measurements performed at a rate of 50 nm/min using a response time of 8 s and a bandwidth of 1 nm. The temperature was kept constant at 25 °C.

2.5. Phospholipase activity assay

Phospholipase-D activity was measured using the Amplex Red Assay Kit (Molecular Probes, Eugene, USA). In this assay, phospholipase-D activity was monitored using 10-acetyl-3,7-dihydroxyphenoxazine (Amplex Red reagent), a sensitive fluorogenic probe for H_2O_2. First, phospholipase hydrolyzes sphingomyelin to yield C1P and choline. Choline is oxidized by choline oxidase to betaine and H_2O_2. Finally, H_2O_2 in the presence of horseradish peroxidase reacts with Amplex reagent in a 1:1 stoichiometry to generate the highly fluorescent product resorufin. Recombinant toxins (10 μg each to start, in three trials; later 100, 250 and 500 μg of LiRecDT1 H12A) were added to the Amplex Red reagent mixture to a final volume of 500 μl. The reaction tubes were incubated at 37 °C in a water bath for 30 min and fluorescence was measured in a fluorometer (Shimadzu Model RF-5301 PC Fluorescence Spectrophotometer) using excitation at 560 nm and emission detection at 590 nm. The controls were LALP (loxosceles astacin-like protease) [32] for a negative control and whole venom for a positive control. Sphingomyelinase (0.004 U) from *Bacillus cereus* (1 U = 1 μmol min^{-1}) was co-tested, and fluorescence intensity was used as standard for comparing ends. Fluorescence intensity values were converted to units of phospholipase activity, which were expressed as μmol min^{-1} (amount of substrate hydrolyzed per minute).

2.6. Animals

Adult rabbits weighing approximately 3 kg obtained from the Central Animal House of the Federal University of Paraná were used for *in vivo* experiments with recombinant toxins. All experimental protocols using animals were performed according to the "Principles of laboratory animal care" (NIH Publication no. 85-23, revised 1985) and the "Brazilian Federal Laws" as well as the ethical committee agreement number 246 of the Federal University of Paraná.

2.7. Dermonecrosis in vivo

For the evaluation of dermonecrotic effects, 10 μg of each toxin was injected intradermally into a shaved area of the rabbit skin for the first experiments. Animals were observed over the course of dermonecrotic lesion evolution. The LiRecDT1 H12A residual activity experiment was performed under the same conditions, except that doses were increased to 100, 250, and 500 μg of mutated toxin. Acquisition of macroscopic pictures was performed after 4 h and 24 h of toxin application, and skin samples were collected at the end of the experiment for microscopic analysis and myeloperoxidase (MPO) activity assay.

2.8. Tissue MPO activity assay

The activity of tissue MPO was also assessed 24 h after injection of toxins into the rabbit skin, according to a technique modified from Bradley et al. [33,34]. Briefly, samples were placed in 0.75 ml of 80 mM phosphate-buffered saline (PBS), pH 5.4, containing 0.5% hexadecyltrimethylammonium bromide, and then homogenized (45 s at 0 °C) in a motor-driven homogenizer. The homogenate was decanted into a microfuge tube, and the vessel was washed with 0.75 ml of hexadecyltrimethylammonium bromide in buffer. The wash was added to the tube, and the 1.5-ml sample was centrifuged at 12,000×*g* at 4 °C for 15 min. Triplicate 30 μl samples of the resulting supernatant was added to 96-well microtiter plates. For the assay, 200 μl of a mixture containing 100 μl of 80 mM PBS, pH 5.4, 85 μl of 0.22 M PBS, pH 5.4, and 30 μl of 0.017% hydrogen peroxide was added to the wells. The reaction was started with the addition of 20 μl 18.4 mM tetramethylbenzidine HCl in dimethylformamide. Plates were incubated at 37 °C for 10 min, and then the reaction was stopped by the addition of 30 μl of 1.46 M sodium acetate, pH 3.0. Enzyme activity was determined colorimetrically using a plate reader (EL808, BioTech Instruments, Inc.) set to measure absorbance at 630 nm and was expressed as mOD/mg tissue.

2.9. Histological procedure for light microscopy

Skin samples were collected from rabbits anesthetized with ketamine (Agribands, Paulinia, Brazil) and acepromazine (Univet, São Paulo, Brazil) and then fixed in Bouin's fixative solution (picric acid saturated solution, 750 ml; formaldehyde 36–40%, 250 ml; acetic acid, 50 ml) for 16 h at room temperature. After fixation, samples were dehydrated in a graded series of ethanol before being paraffin embedded (for 2 h at 58 °C). Then, thin tissue sections (4 μm) were processed and stained with hematoxylin and eosin (H&E).

2.10. Immunological cross-reactivity of original and mutated toxin

The protein content of experimental samples was determined by the Coomassie Blue method (BioRad, Hercules, USA). For immunoblotting, 5 μg of each toxin was subjected to 12.5% SDS-PAGE under reducing conditions, transferred onto nitrocellulose filters overnight and immunostained with hyperimmune sera (either against phospholipase-D toxin or whole venom). Secondary alkaline phosphatase-coupled anti-IgG were detected and visualized using the BCIP/NBT substrate reaction. For the antibody capture assay (ELISA), tests were performed in pentaplicate with 10 μg/ml of venom and LiRecDTs toxins as antigens. Purified hyperimmune IgGs, anti-LiRecDT1, and anti-whole venom were tested (0.1–1 μg/ml), followed by detection with secondary peroxidase-coupled anti-IgG and reading of the absorbance at 450 nm for the OPD substrate reaction [35].

2.11. Protein–lipid overlay assay (fat blot)

The affinity of toxins for specific lipids was tested using the SphingoStrips recommended method (Molecular Probes, Eugene, USA). In summary, nitrocellulose membranes containing 100 pmol samples of 15 different lipids and a blank sample were blocked using Tris-buffered saline plus 0.02% Tween-20 (TBS-T) with 3% fatty acid-free bovine serum albumin (BSA) and gently agitated for 1 h at room temperature. Then, the membranes were incubated with 0.5 μg/ml of the toxins (whole venom, LiRecDT1, or LiRecDT1 H12A) in TBS-T plus 3% BSA at 4 °C overnight. Membranes were then washed with TBS-T plus 3% BSA three times for 10 min using gentle agitation. Toxin binding was detected using anti-venom or anti-LiRecDT1 purified IgGs and revealed by alkaline phosphatase-labeled secondary antibodies and BCIP/NBT substrate.

2.12. Cell culture conditions

RAEC (rabbit aorta endothelial cells) were grown as monolayer cultures in F12 medium containing gentamicin (40 mg/L) supplemented with 10% fetal calf serum (FCS). The cultures were kept at 37 °C in a humidified atmosphere with 5% CO_2. For inverted light microscopy observation and confocal immunofluorescence analysis, release of cells was performed by treatment with 0.25% viokase/HBSS for a few minutes. After counting, the cells were resuspended in an adequate volume of medium supplemented with FCS, and 5×10^3 cells were replated on glass coverslips (13 mm diameter) and allowed to adhere and grow for 48 h. The cells were then incubated in the presence of the recombinant toxins (10 μg/ml). Changes in cell morphology were evaluated and photographed using an inverted microscope (Leica-DMIL, Wetzlar, Germany) at 1 h and 4 h after recombinant toxin addition. The same experimental conditions were used with control cells, except that the medium contained appropriate amounts of vehicle (PBS) instead of recombinant toxins.

2.13. RAEC immunofluorescence assay

For immunofluorescence microscopy, after recombinant toxins exposure for 4 h at 37 °C, RAEC cells were rapidly washed with PBS, fixed with 2% paraformaldehyde in PBS for 30 min at 4 °C, incubated with 0.1 M glycine for 3 min, and blocked with PBS containing 1% BSA for 1 h at room temperature. Coverslips were incubated for 1 h with polyclonal antibodies raised against phospholipase-D (2 μg/ml) as described in the Reagents section. Cells were washed three times with PBS, blocked with PBS containing 1% BSA for 30 min at room temperature, and then incubated with Alexa® 488-conjugated anti-rabbit IgG secondary antibodies (Molecular Probes) at room temperature for 40 min. After washing, samples were mounted using Fluormont-G (Sigma) and observed under a fluorescence confocal microscope (Confocal Radiance 2100, BioRad) coupled to a Nikon-Eclipse E800 with Plan-Apochromatic objectives (Sciences and Technologies Group Instruments Division, Melville, USA).

2.14. Choline release detection from RAEC cell extract

Extracts of RAEC cell membranes were obtained from approximately 5×10^6 cells (150 cm^3 culture bottle). Cells were scraped and resuspended in 1 ml of cold extraction buffer (Tris–HCl 50 mM, NaCl 150 mM, Triton X-100 0.5%). After gently homogenizing for 10 min at 4 °C, cells were centrifuged at $20{,}000\times g$ for 20 min at 4 °C and supernatants were collected for later use. RAEC cells extracts (100 μl) were utilized as a substrate for LiRecDT1 and LiRecDT1 H12A (both in two concentrations, 50 μg and 100 μg) in a total final volume of 250 μl for 1 h at 37 °C and gently mixed using a rotational shaker in a BOD incubator. Treated extracts were then added to a 250 μl reaction mixture adapted from the Amplex Red Sphingomyelinase Assay Kit (Molecular Probes) containing choline oxidase (4 U), alkaline phosphatase (80 U), horseradish peroxidase (20 U), and Amplex Red reagent (100 μM), excluding sphingomyelin substrate. After incubation in a water bath for 1 h at 37 °C, fluorescence development was measured in a fluorometer (Shimadzu Model RF-5301 PC Fluorescence Spectrophotometer) using excitation set at 560 nm and emission detection at 590 nm.

2.15. Lipid analysis by high-performance thin-layer chromatography

For analysis of hydrolysis, recombinant toxins (50 μg) were incubated with RAEC cell extracts (50 μl or 100 μl) for 3, 8 or 16 h, and purified phospholipids for 3 h at 20 °C. Lipids present in these detergent-soluble samples were recovered directly by partition with 2 ml of water-saturated 1-butanol; the butanol fraction was dried, resuspended in chloroform, and analyzed by high-performance thin-layer chromatography (HPTLC). Analytical HPTLC was performed on silica gel 60 plates (E. Merck, Darmstadt, Germany) using chloroform–methanol–methylamine 40% (65:35:10 v/v/v) as the mobile phase. Lipid samples were dissolved in chloroform and 20 μl were applied by micropipetting, and then visualized under ultraviolet light after spraying with 0.01% primulin in 90% aqueous acetone [36]. Differences in lipid content were quantified by densitometry of digital images from HPTLC plates acquired by GeneSnap Software for G:Box Chemi XL (Syngene, Cambridge, England) and quantified by Quantity One Software for Chemic Doc XRS (BioRad, Hercules, USA). The standard mixture contained 1 mg/ml each of five phospholipids (Sigma): phosphatidylinositol (PI), phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylserine (PS). Toxins were also incubated with 10 μg of purified phospholipids: asymmetric PC or 16:0–18:0 PC (1-palmitoyl-2-stearoyl-*sn*-glycero-3-phosphocholine), and PAF *platelet-activating factor* or C16-2:0 PC (1-O-hexadecyl-2-acetoyl-*sn*-glycero-3-phosphocholine), and synthetic alkyl-phospholipids such as *lyso*PC or 16:0 Lyso PC (1-palmitoyl-2-hydroxy-*sn*-glycero-3-phosphocholine) and *lyso*PAF or C16 Lyso PAF (1-O-hexadecyl-2-hydroxy-*sn*-glycero-3-phosphocholine). Purified egg chicken sphingomyelin (egg SM) and other phospholipids tested were purchased from Avanti Polar Lipids, Inc. (Alabaster, USA).

2.16. Statistical analysis

Statistical analysis of phospholipase activity and choline release data were performed using analysis of variance (ANOVA) and the Tukey test for average comparisons GraphPad InStat program version 3.00 for Windows. Mean ± SEM. values were used. Significance was determined as $p \leq 0.05$.

3. Results

3.1. Cloning and expression of a mutated dermonecrotic toxin from LiRecDT1: drastic decrease of phospholipase-D activity by a single amino acid mutation

In order to obtain a mutated form of *L. intermedia* dermonecrotic toxin, an adapted "Megaprimer" PCR approach for site-specific mutation was used to clone a mutated dermonecrotic toxin based on the LiRecDT1 (GenBank accession number ABA62021) amino acid sequence [12]. This recombinant toxin contained an amino acid sequence that was substituted with alanine at the first histidine residue (His12), which plays a role in the catalytic reaction. Alteration of the active site was confirmed by sequencing analysis, and the resulting LiRecDT1 H12A mutated recombinant toxin was successfully expressed in the pET-14b system and purified in soluble form by Ni^{2+}-NTA affinity chromatography (Fig. 1A). To insure the correct folding of the mutated toxin occurred despite the changed nucleotide sequence, which codes for a different protein sequence, conformational circular dichroism spectra measurements were performed. Indeed, we observed a secondary structure spectrum for the mutated form that was very similar to the LiRecDT1 CD profile as depicted in Fig. 1B. To investigate the enzymatic behavior of the mutated LiRecDT1 H12A phospholipase-D toxin *in vitro*, a fluorometric assay using sphingomyelin as the reaction substrate was performed (see Materials and methods section for details). LiRecDT1 and whole venom showed the expected high phospholipase-D activity [12], but the mutated toxin had almost no sphingomyelin hydrolysis activity under the same conditions. Fig. 1C shows that the activity of 10 μg of recombinant original toxin was about 85-fold higher than that of the mutated toxin.

3.2. LiRecDT1 H12A did not induce dermonecrosis in rabbit skin and caused only a small inflammatory reaction

Dermonecrotic macroscopic development was observed in rabbit skin after 4 h and 8 h of exposure to 10 μg of whole venom (as a positive control) and original recombinant dermonecrotic toxin. However, treatment with the mutated form did not cause obvious dermonecrotic development, and the reaction was similar to the negative control (see Experimental). In addition, no edema or erythema was observed at the injection site in response to the mutated form (Fig. 2A). Light microscopic analysis of rabbit skin biopsies after 24 h of toxin treatment (Fig. 2B) demonstrated typical intense inflammatory cell accumulation and collagen fiber disorganization in the dermis with LiRecDT1 treatment. However, biopsy analysis of LiRecDT1 H12A exposed skin demonstrated a less intense inflammatory response. The neutrophil migration into the dermis was indirectly determined by means of MPO activity of tissue biopsies (Fig. 2C), and the differences in these values correlate with the microscopic analysis of inflammatory reaction.

3.3. The residual activity of LiRecDT1 H12A is concentration-dependent

As the microscopic analysis revealed an inflammatory reaction in rabbit skin treated with mutated recombinant toxin, it is possible that very low phospholipase-D activity persists despite modification of His12A in the catalytic site. This residual activity could be responsible for the observed biological effects. For this reason, phospholipase-D activity and dermonecrosis analysis of LiRecDT1 H12A were examined again at much higher concentrations. Higher amounts of LiRecDT1 H12A (100 μg, 250 μg, and 500 μg) were used in both experiments with the previously described conditions (Fig. 3). A 2.4-fold increase in phospholipase activity was observed between 100 and 500 μg protein. The phospholipase-D activity of the highest concentration tested (500 μg) compared to 10 μg of original recombinant toxin was still 7-fold less intense (Figs. 1C and 3A). Moreover, Fig. 3B depicts *in vivo* experiments showing formation of edema and erythema at the injection site that were much less intense compared to those observed with LiRecDT1 after 4 h. At 24 h post LiRecDT1 H12A exposure, rabbit skin showed more edema, and an initial white eschar, known as a marble plate in loxoscelism (that commonly evolves into necrotic lesion) could be seen. Microscopic analyses (Fig. 3C) of biopsies from

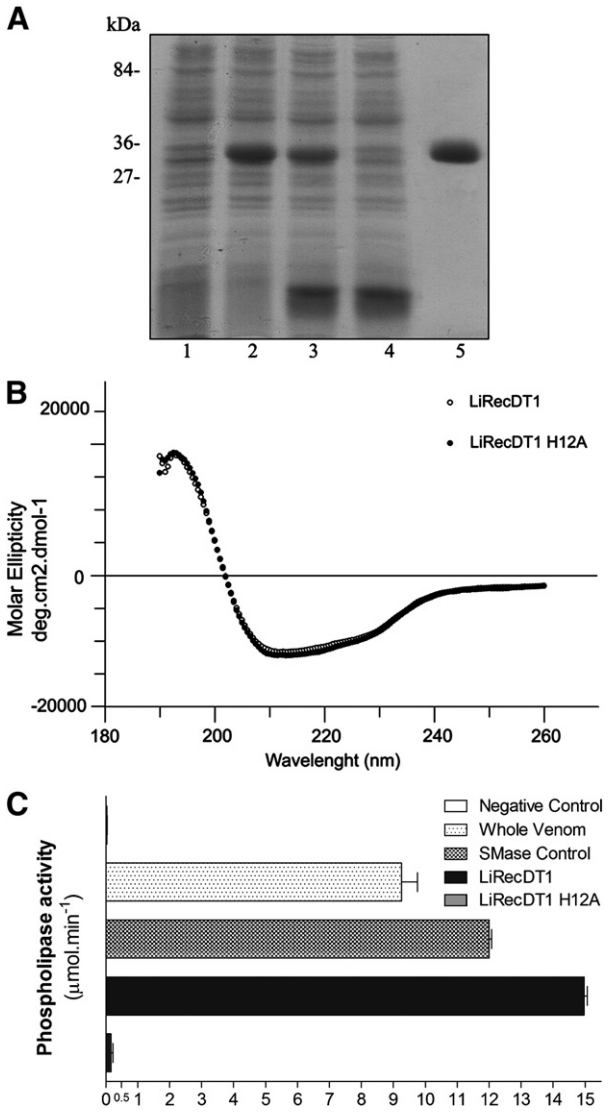

Fig. 1. Expression of a functional recombinant site-directed mutated dermonecrotic protein (LiRecDT1 H12A). (A) SDS-PAGE analysis of recombinant dermonecrotic toxin expression stained with Coomassie blue dye (12.5% gel under reducing conditions). Lanes 1 and 2 show *E. coli* BL21(DE3)pLysS cells collected by centrifugation and resuspended in gel loading buffer before and 3.5 h after induction with 0.05 mM IPTG, respectively. Lanes 3 and 4 depict the supernatant of cell lysates obtained by freeze thawing in extraction buffer before and after (*void*) incubation with Ni-NTA agarose beads, respectively. Lane 5 shows eluted recombinant protein. Molecular masses are shown on the left. (B) Circular dichroism spectra for the purified recombinant original and mutated toxins. Spectra were obtained using toxins in 10 mM sodium phosphate buffer, pH 7.4, at 25 °C. Molar ellipticity was analyzed in a wavelength range between 180 and 260 nm. (C) Phospholipase-D activity of recombinant toxins. Sphingomyelinase activities of LiRecDT1, LiRecDT1 H12A recombinant toxins, and whole venom were evaluated with the Amplex Red Assay Kit at 37 °C for 1 h; the product of the reaction was spectrofluorimetrically measured at 560 nm excitation and 590 nm emission wavelengths. A purified recombinant toxin without dermonecrotic and inflammatory activities was used as a negative control. Reactions were performed with 10 μg of whole venom or recombinant toxins ($n = 6$). Values given are the average ± SEM.

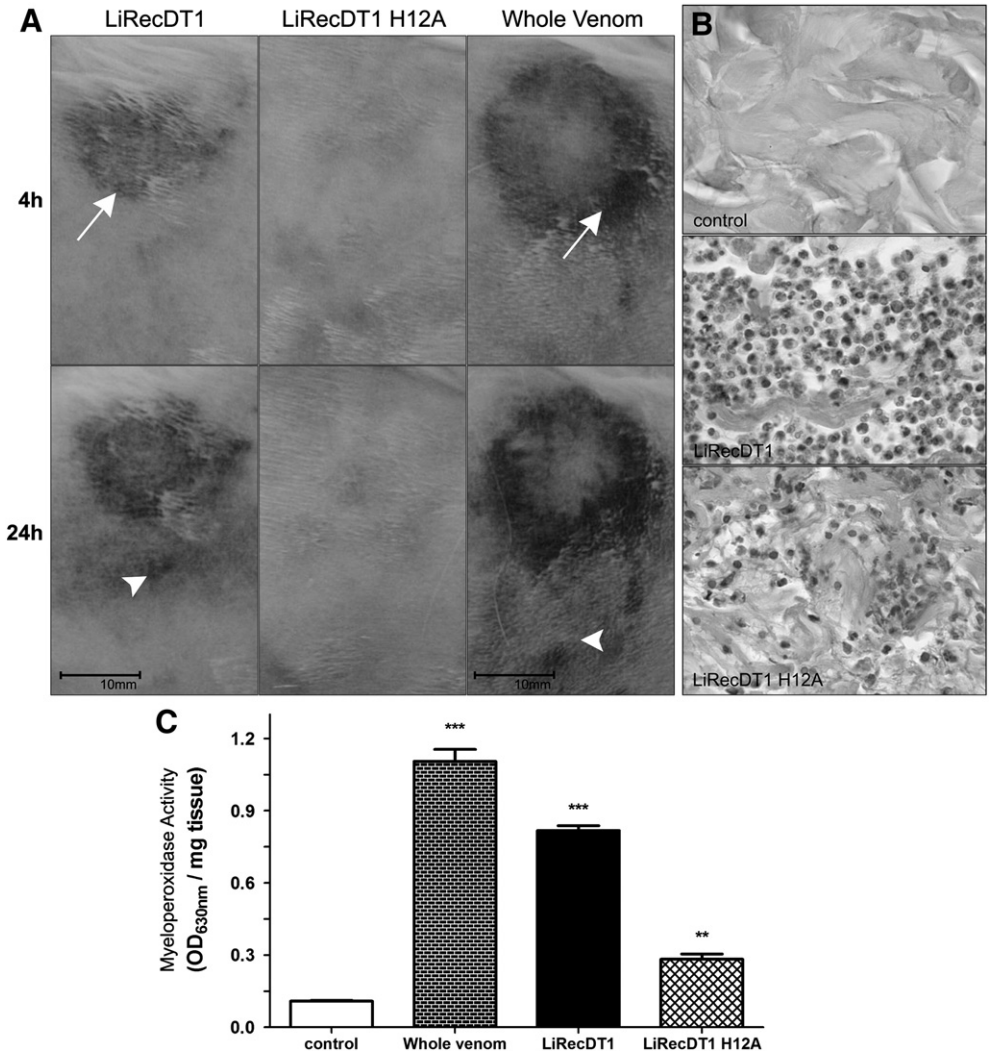

Fig. 2. Dermonecrosis and inflammatory response of rabbit skin exposed to recombinant toxins. (A) Macroscopic visualization of dermonecrosis of rabbits intradermally injected with 10 μg of whole venom (as positive control) or both recombinant toxins. Lesions were observed 4 and 24 h following venom and recombinant toxin injections. Arrows point to development of gravitational spreading of necrotic lesion post LiRecDT1 and whole venom injections. The scale bar is shown on the left. (B) Histopathological findings for rabbit skin were performed 24 h after recombinant toxin exposure. Light microscopic analysis of tissue sections were assessed by staining with hematoxylin and eosin (H&E). An intense inflammatory response with the presence of neutrophils and disorganization of collagen fibers in connective tissue is shown for the original toxin compared to a lower inflammatory reaction observed following mutated recombinant dermonecrotic toxin exposure. A recombinant toxin without phospholipase-D activity but obtained under identical conditions was used as a negative control (magnification 630×). (C) Inflammatory reactions induced by toxins and controls were estimated by measurement of myeloperoxidase activity from neutrophil infiltrate at the dermis. Values are expressed as mean ± SEM of absorbance at 630 nm. Each bar represents the mean of fifteen replicates (five from each of three tissue samples) of inoculation site at rabbit skin post 24 h. The asterisk denotes the significance levels when compared with negative control (*** $p<0.001$; ** $p<0.01$).

rabbit skin treated with LiRecDT1 H12A at the higher concentrations showed an abundant neutrophil perivascular infiltrate and collapse of blood vessel walls into the dermis.

3.4. Immunological cross-reactivity of original and mutated LiRecDTs

In order to determine whether antigens of our antibodies are conserved in the recombinant LiRecDT1 and LiRecDT1 H12A, we produced polyclonal antisera against whole venom and LiRecDT1. Whole venom served as the positive control for native phospholipase-D toxins, which were also recognized by both antisera. Experiments were performed by Western blot (antibodies recognize epitopes on denatured antigens) and ELISA (antibodies recognize non-denatured epitopes on antigens). The cross-immunoreaction of antisera for both recombinant toxins showed that site-directed mutation did not alter immunological recognition by polyclonal antibodies; therefore, these antibodies can be used for further experiments (Fig. 4).

3.5. Binding of both recombinant toxins to immobilized lipids in an overlapping assay

Fat blot assays are used for the identification of proteins possessing lipid recognition domains and for analysis of their lipid-binding specificities. Several different toxin interactions were detected via the protein–lipid overlay assay with a standard Western blot. These interactions have been shown to be involved in physiological and pathological processes including signal transduction mediated by GPCRs and induction of apoptosis [18–20,37,38]. With the goal of determining whether dermonecrotic toxin possesses some lipid affinity, we exposed the original and mutated isoform to immobilized lipids (Fig. 5). Additionally, we tested whole venom as a control to test native dermonecrotic toxins. Both recombinant isoforms bound to the same molecules: sphingomyelin and sphingosine 1-phosphate, phosphatidylcholine and *lyso*phosphatidylcholine, and cholesterol. As a negative control, the membrane containing immobilized lipids was exposed to antibodies in the absence of toxins (data not shown).

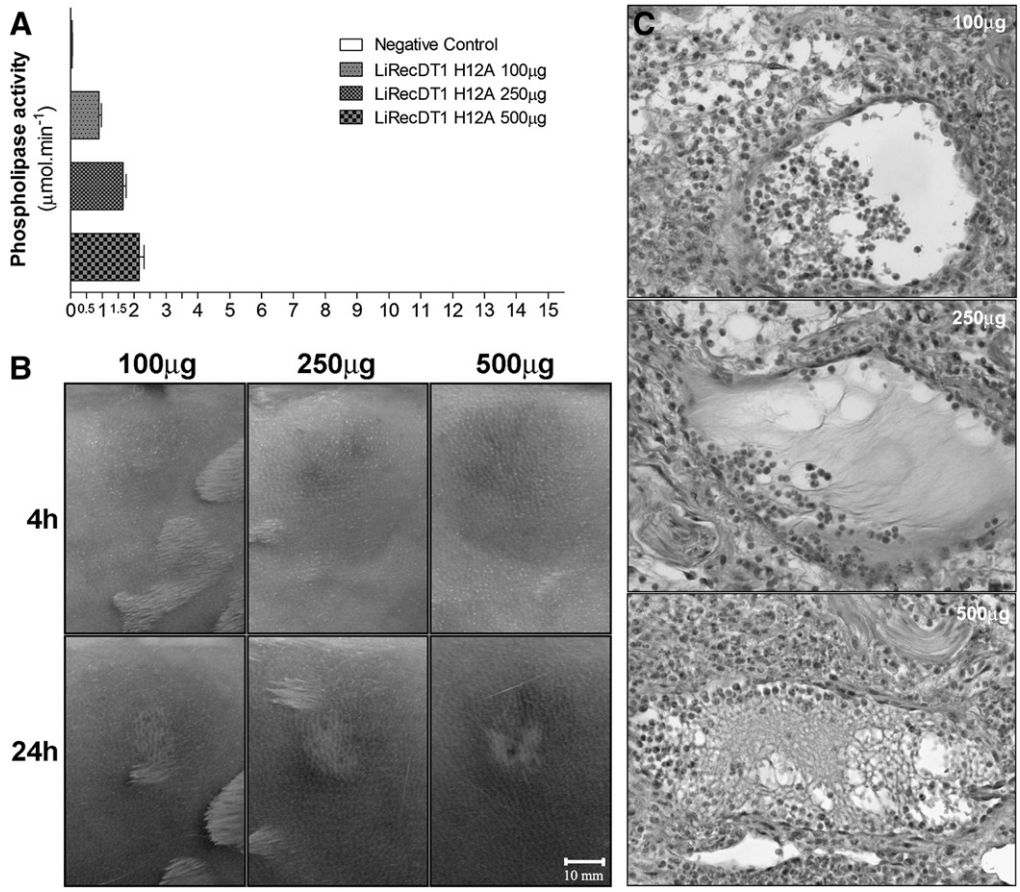

Fig. 3. Residual phospholipase-D and inflammatory activities of LiRecDT1 H12A. (A) Mutated recombinant toxin was tested for persistent sphingomyelinase activity with increasing doses of 100, 250, and 500 μg. Then, the Amplex Red Sphingomyelinase Assay Kit assay was performed using the same conditions and incubating at 37 °C for 1 h. Reactions were measured in a spectrofluorometer (wavelengths of 560 nm for excitation and 590 nm for emission). As a negative control, 10 μg of enzymatic non-related recombinant toxin was also tested to assess background fluorescence. Increasing concentration-dependent phospholipase-D activity was observed ($n=6$). Values given are the average ± SEM. (B) Macroscopic analysis of rabbit skin exposed to higher concentrations of LiRecDT1 H12A. In a dose-dependent reaction, we observed a punctate and transient erythema at the injection site 4 h post exposure to mutated toxin exposure (100, 250, and 500 μg). After 24 h, development of an ischemic halo surrounding the injection site indicates typical and initial formation of dermonecrosis described as a marble plate, and (C) microscopic analysis of tissue sections by H&E show a large inflammatory response with massive neutrophil diapedesis and fibrin deposition at disrupted blood vessels for all three concentrations tested (magnification 630×).

The H12A substitution in the catalytic domain did not alter LiRecDT1 binding-affinity to the lipids.

3.6. Recombinant dermonecrotic toxins bind to the RAEC cell surface as "planted antigens"

We next examined the cell membrane interaction of the dermonecrotic toxin phospholipase-D family specifically, as whole venom is a complex mixture of biochemically and biologically different toxins. To do this, we evaluated recombinant dermonecrotic toxin binding to the endothelial cell surface using hyperimmune IgGs against LiRecDT1 by confocal immunofluorescence analysis (Fig. 6). Cells treated with both recombinant toxins showed an intense positive reaction on the cell surface. Untreated cells (negative control) were treated to confirm that there was no non-specific binding of the hyperimmune antibodies. The interaction of toxins with RAEC cell membranes had a homogeneous distribution pattern. Again, LiRecDT1 H12A was also able to bind to the plasma membrane (an environment enriched in lipids) despite the mutation in the catalytic site.

3.7. Choline release from RAEC cell membrane extracts with LiRecDT1 treatment

Since the appearance of cluster-like aggregates were observed on RAEC cell membranes only after LiRecDT1 but not mutated toxin treatment, we examined whether cell membrane RAEC extracts could act as an enzymatic substrate for phospholipid hydrolysis. The original substrate used for the sphingomyelinase activity kit test (a purified sphingomyelin) was substituted with cell membrane detergent extract obtained from RAEC cells in culture (see Materials and methods for details). Therefore, not only sphingomyelin, but also other phospholipids from RAEC cell extracts could be the target of phospholipase-D. Incubation of recombinant toxins with extracts triggered significant formation of choline in response to LiRecDT1 in a concentration- and time-dependent manner (Fig. 7A). Changes in cell morphology triggered by LiRecDT1, such as cytoplasmic vacuolization and the presence of debris resulting from cell lysis were observed (Fig. 7B). These results suggested that phospholipase-D activity on membrane phospholipids correlates directly with cytotoxicity.

3.8. LiRecDT1 degrades lipids from RAEC cell membrane extracts and purified phospholipids

In addition to the phospholipase-D activity indirectly shown by choline release, hydrolysis of RAEC cell membrane extracts can be observed through high-performance thin-layer chromatography (HPTLC). Fig. 8A and B shows that after incubation with both LiRecDT1 and LiRecDT1 H12A, the intensity of primuline-positive bands decreased compared to non-treated extract control. With 50 μl of extracts, the band for phosphatidylethanolamine was gradually reduced to a final relative quantity of 37% after 16 h with active phospholipase-D. However, the mutated isoform was able to degrade

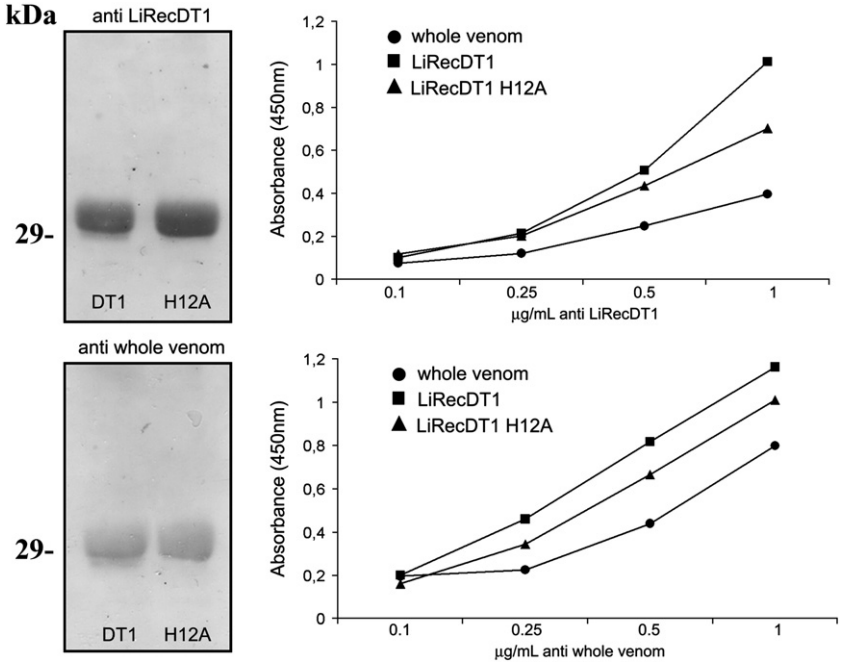

Fig. 4. Immunological cross-reactivity of the recombinant toxins LiRecDT1 and LiRecDT1 H12A. Original (DT1) and mutated (H12A) toxins at a concentration of 10 µg were electrophoresed by 12.5% SDS-PAGE under reducing conditions, transferred to nitrocellulose membranes, and incubated with purified IgGs from sera recognizing whole venom or LiRecDT1. Immunoblotting was performed with secondary antibodies coupled to alkaline phosphatase and BCIP/NBT substrate. Additionally, antibody capture assays (ELISA) were carried out using whole venom and recombinant toxins LiRecDT1 and LiRecDT1 H12A (10 µg/ml) immobilized on a solid phase. Again, purified antibodies against whole venom or LiRecDT1 (abscissa) were incubated for 2 h at room temperature and the immunoreaction was performed using secondary antibodies coupled to peroxidase and OPD substrate. Colorimetric measurement was performed by absorbance at 450 nm. Values given are average of pentuplicates.

only 5% of initial content. With two-fold greater volume (100 µl) of extracts but the same concentrations of recombinant toxins, bands for other lipids such as sphingomyelin and phosphatidylcholine are visible and show drastic intensity decreases. Specifically, SM was reduced to 5.6% and PC to 13.0% of the control. Interestingly, the mutated isoform of phospholipase-D was able to significantly degrade SM after 16 h to less than a half of initial content (41.1%), but the same was not observed for PC, which was only reduced to approximately 87.2% of control. The bands for phosphatidylserine were also reduced by both toxins, to 58.9% and 63.2% of control for LiRecDT1 and LiRecDT1 H12A, respectively. Next, we tested purified phospholipids individually (Fig. 8C and D) and found that 3 h after incubation, egg sphingomyelin and *lyso*PC were most susceptible to hydrolysis by recombinant toxins from *L. intermedia* with a reduction to 6.2% and 4.9% of control, respectively. The H12A mutation did not completely abolish its enzymatic ability, as the mutated toxin hydrolyzed both phospholipids but with lower effectiveness (77.0% and 59.2%, respectively). In addition, PC asymmetric, PAF and lysoPAF were also degraded by the toxins, but more significantly by the original than the mutated isoform. These results suggest that brown spider phospholipase-D has no specificity for one single type of lipid as a substrate/target. Lipid-derived products can be visualized at the top of those lanes with intense decrease of initial lipid content (*). Purified sphingomyelin and lysophospholipids were the best substrates of this enzyme, but after only 3 h of incubation other non-lysophospholipids were also significantly degraded. These were also observed in HPTLC with cell membrane extracts.

4. Discussion

Loxoscelism is characterized as a group of severe clinical symptoms triggered by brown spider (*Loxosceles* genus) bites. Brown spider bites can induce dermonecrotic lesions, hematological disorders, and renal failure [1,2]. Histopathological findings for rabbit skin experimentally

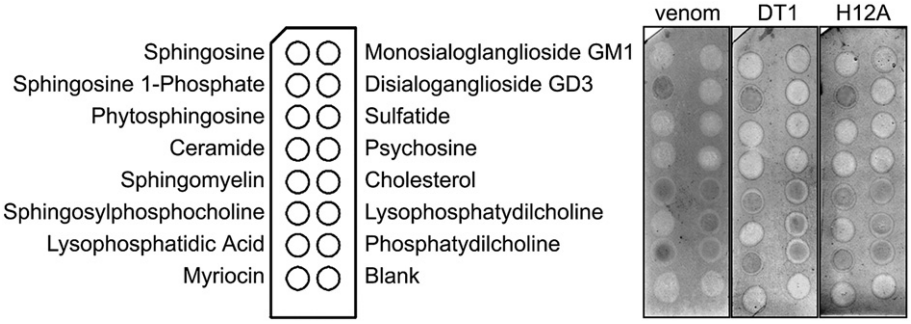

Fig. 5. Lipid binding of venom toxins in fat blot assays. Each spot contains 100 pmol of the indicated lipid in the layout of strips used to test the affinity of whole venom and recombinant toxins, which were incubated at a concentration of 0.5 µg/ml with the membranes. Samples of whole venom, LiRecDT1, and LiRecDT1 H12A show overlapping with the same spotted lipids: sphingosine 1-phosphate and sphingomyelin, lysophosphatidic acid, cholesterol, and both *lyso*phosphatidylcholine and phosphatidylcholine. Lipid binding of the toxins was detected using purified IgGs against whole venom or against LiRecDT1, and revealed with secondary antibodies coupled to alkaline phosphatase and BCIP/NBT substrate.

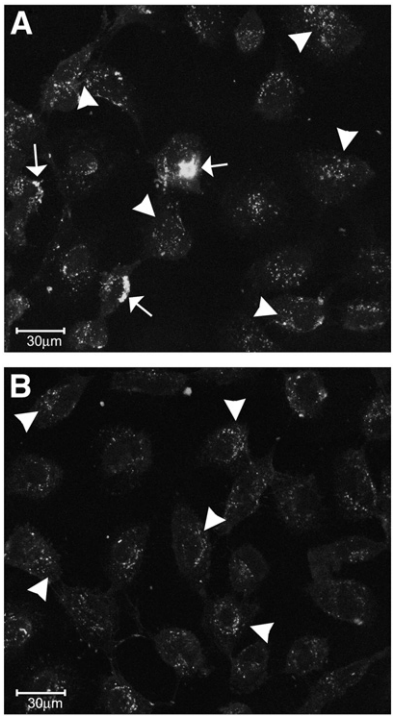

Fig. 6. Recombinant dermonecrotic toxin binding to RAEC cell membranes. Confocal immunofluorescence microscopy analysis was performed for both LiRecDT1 and LiRecDT1 H12A-treated endothelial cells incubated with antibodies anti-LiRecDT1 and a specific secondary fluorescent conjugate. (A) Four hours post-incubation of Original toxin (40 μg/ml), the cell surface showed punctate binding of LiRecDT1 (arrowheads) and deposition as aggregated clusters (arrows). (B) LiRecDT1 H12A treatment (40 μg/ml) also caused non-homogeneous binding of the mutated toxin (arrowheads), but no cell membrane aggregation was visualized. The scale bar is visualized on the left.

exposed to whole venom have shown the degeneration of blood vessel walls, endothelial cytotoxicity, and fibrin and thrombus formation [6,7]. Likewise, capillary hyperpermeability and intense infiltration of polymorphonuclear leukocytes around blood vessels cause a massive inflammatory response to the venom toxins [2,11].

Brown spider venom is a complex mixture of biochemically and biologically varied toxins reported by proteomic analysis to have molecular masses predominantly in the range of 3–40 kDa [39–41]. Most reported biological effects can be reproduced by dermonecrotic toxin family members (phospholipases-D). Previous reports have shown that a recombinant dermonecrotic toxin from the *L. intermedia* venom gland (LiRecDT1) was capable of stimulating dermonecrosis, hemolysis, platelet aggregation, renal disturbance, increased vascular permeability, and a deregulated inflammatory activation [11–13,15,41].

Futrell [2] has characterized dermonecrotic toxin as a sphingomyelinase-D enzyme because of its ability to hydrolyze sphingomyelin into choline and acylsphingosine phosphate. Recently, Lee and Lynch [10] suggested the term sphingomyelinase-D should be replaced by phospholipase-D to better classify brown spider dermonecrotic toxins based on their additional ability to hydrolyze glycerophospholipids. As such, the formation of lipid metabolites (also known as potent bioactive molecules) such as C1P or LPA could be responsible for the toxicity by activating signaling pathways related to a variety of pathophysiologic changes [10,18,20].

In this work, we investigated the dependence of the enzymatic activity of phospholipase-D to evoke the toxicity displayed in response to the biological effects of brown spider dermonecrotic toxin. To this end, we produced a mutated form of the dermonecrotic toxin from *L. intermedia* venom. The previously described LiRecDT1 was modified at the catalytic site with a single amino acid change using a site-directed mutagenesis strategy. In the literature, several studies have used the mutation of specific amino acid residues to evaluate the structure and function of toxins [42,43]. Kukreja et al. [44] examined the molecular action of botulinum neurotoxin endopeptidase by substituting Glu262 with Asp and showed a 3-fold decrease in catalytic efficiency. This mutation did not induce any significant structural alterations in the active site and did not interfere with substrate binding.

Based on a study of the crystal structure of *L. laeta* dermonecrotic toxin (SMaseI) [17], the proposed acid base catalytic mechanism postulated residues His12 and His47 as playing key roles and being supported by a network of hydrogen bonds between Asp34, Asp52, Trp230, Asp233, and Asn252. We chose the first histidine residue (His12) in the catalytic site as a mutation target, intending to abolish phospholipase-D activity of the resulting toxin.

Recombinant expression of the mutated toxin as a fusion protein was successfully achieved (Fig. 1A and B) and we named it LiRecDT1 H12A (*Loxosceles intermedia* dermonecrotic toxin 1, mutation at His12 to Ala residue). To confirm the effectiveness of the site-directed mutation in extinguishing phospholipase-D activity, we performed classic tests for characterizing dermonecrotic toxin: sphingomyelin hydrolysis *in vitro* and dermonecrosis *in vivo* [1,2]. Compared to 10 μg of the original toxin and whole venom, LiRecDT1 H12A presented insignificant phospholipase-D activity and no macroscopic effect on rabbit skin (Fig. 1C). Meanwhile, histopathological findings for the LiRecDT1 H12A tissue samples showed the presence of a neutrophil infiltrate and collagen fiber disorganization in the treated rabbit skin (Fig. 2B). Despite this controversial finding, we presume that LiRecDT1 H12A did not lose phospholipase-D activity completely. Indeed, residual enzymatic activity was observed by testing higher concentrations of the mutated toxin (Fig. 3). The phospholipase-D activity of 500 μg of the mutated toxin increased compared to 10, 50, and 100 μg, but was considerably lower than 10 μg of the original toxin, suggesting some residual catalytic activity of the protein.

Cross-reactivity experiments demonstrated that antibodies against venom or LiRecDT1 can also recognize linear (Fig. 4) and conformational epitopes (Fig. 4) of LiRecDT1 H12A. As a result, the mutated toxin could be used as a biological tool for comparing identical proteins with almost insignificant enzymatic activity that is a structural analogue for dermonecrotic toxin. First, anti-toxin hyperimmune IgGs were used to reveal the binding of LiRecDT1 to specific lipids immobilized in nitrocellulose (Fig. 5). Protein–lipid overlapping experiments are *in vitro* reproductions of biological events triggered by protein interactions with lipid domains on the plasma cell membrane. Recombinant original and mutated toxins bound to the same lipids, including sphingomyelin and phosphatidylcholine.

The His12Ala modification in the amino acid sequence of recombinant dermonecrotic toxin did not interfere with its lipid-binding property. LiRecDT1 may be interacting by a linear or conformational region in the molecule independently of catalytic activity or even the active site domain. It has been well described that proteins expose hydrophobic surfaces of their molecular structures, allowing them to bind phospholipids on the cell membrane and trigger various biological effects [45,46]. LiRecDT1 contains 42.6% hydrophobic residues in its amino acid sequence. Several short sequences show conserved hydrophobicity in LiRecDT1 (AILMFPWYV) and *L. laeta* SMase I (AY093599) based on amino acid sequence alignment. Structural analysis of recombinant dermonecrotic toxin from SMase I [16,17] has shown that hydrophobic loops participate in the interfacial region of the enzyme where the active site is located in a shallow cleft.

Hydrophobic domains in the secondary and tertiary structure of brown spider dermonecrotic toxin probably play a role in its ability to bind lipids on the cell membrane independent of enzymatic activity but not necessarily unrelated to the active site of LiRecDT1. Metal-coordination and charged residues in the catalytic domain must be important for targeting phospholipids in a hydrophilic environment

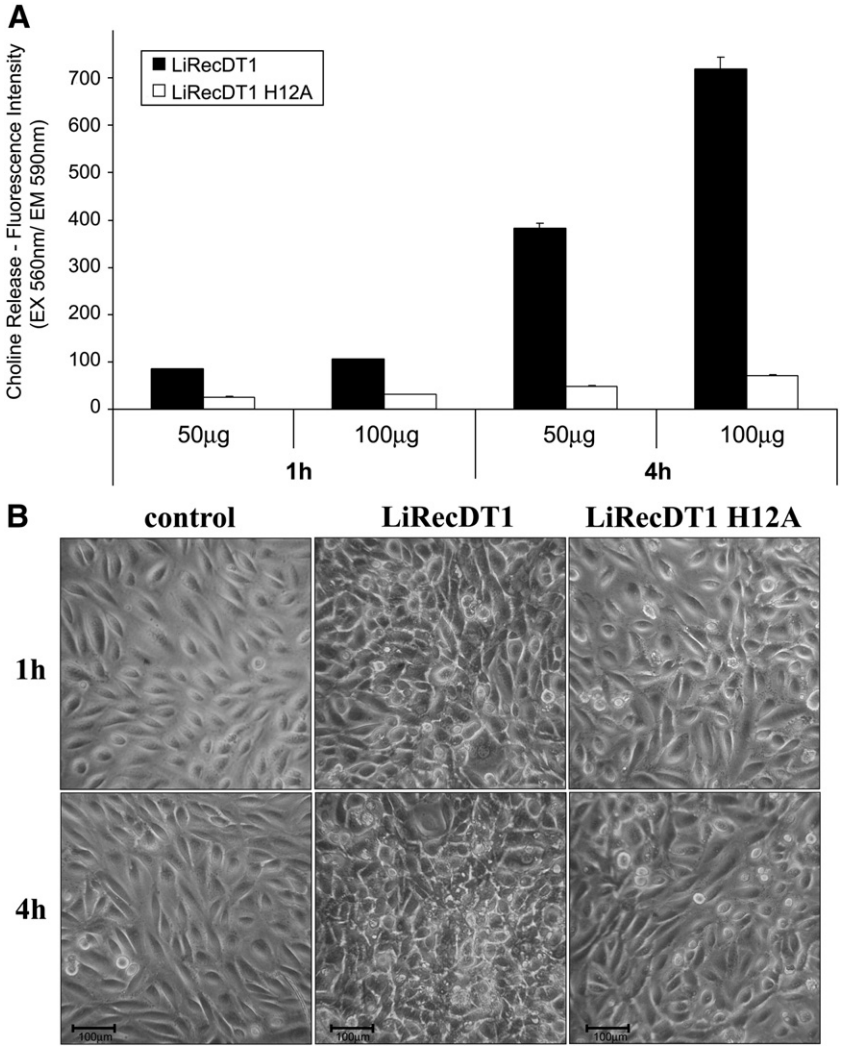

Fig. 7. Choline release induced by recombinant toxins from hydrolysis of membrane extracts as well as cytotoxic effects on endothelial cell cultures. (A) Detergent-extracts of RAEC cell membranes (100 µl) were incubated with LiRecDT1 or LiRecDT1 H12A (abscissa depicts both toxins used at concentrations of 50 µg or 100 µg) for 1 h and 4 h in a 37 °C water bath. Choline formation was detected using a reaction mixture adapted from the Amplex Red Sphingomyelinase Assay Kit (see Materials and methods). Degradation of cell membrane extracts by toxins results in indirect detection of a fluorescent product that was measured in a spectrofluorometer using an excitation at 560 nm with emission detection at 590 nm. (B) Using inverted light microscopy, morphological changes in RAEC cells were observed following incubation of recombinant toxins (10 µg/ml) and images were captured at 1 h and 4 h after exposure. First, LiRecDT1 triggered vacuolization of the cell cytoplasm, which is accompanied by cell lysis and alteration of cellular adhesion to other neighboring cells and substrate. Despite this, LiRecDT1 H12A does not seem to induce a significant change in treated-cells. Control cells, which received adequate amounts of vehicle (PBS) instead of recombinant toxins, displayed typical RAEC cell morphology throughout the experimental procedure.

and are indispensable for hydrolysis of membrane lipids. For example, a prediction for the interaction of alpha-toxin (a phospholipase C from *Clostridium perfringens*) with cell membranes has revealed two different domains: calcium mediated recognition of phospholipids head groups at the active site (amino-terminal region) and interaction of hydrophobic amino acids with the phospholipid tail group. These domains are structurally similar to phospholipid-binding domains in eukaryotic proteins (carboxy-terminal region) [47].

Structure function analysis of LiRecDT1 H12A supports this hypothesis because the same lipid affinity was observed via the fat blot assay despite almost insignificant enzymatic activity. The binding of both toxins was also maintained as demonstrated in treated RAEC cells (as "planted antigens") and immunofluorescence experiments. Recently, in biological approaches with recombinant toxin treated mice, Kusma et al. [28] showed that a mutated isoform binds to intrinsic renal structures as well as the LiRecDT1.

The anti-toxin antibody assays showed the same non-homogeneous pattern for the interaction of both toxins with the RAEC cell surface (Fig. 6). Both recombinant toxins seem to be aggregated throughout the RAEC surface, binding to specific microdomains in the cell membrane. A number of toxins such as bacterial and viral pathogens are able to exploit cholesterol and/or lipid rafts to gain a "foot hold" on their target hosts [48]. Leukotoxin (Ltx) is a bacterial protein toxin from *Actinobacillus actinomycetemcomitans* that induces Ca^{2+} fluxes, mobilizing clusters of LFA-1 (surface antigen of innate and adaptive immune cells) to lipid rafts. Ltx utilizes the raft to stimulate an integrin signaling pathway that results in target cell responses that mimic 'outside-in' activation signals, leading to apoptosis of target cells [49] but no change in lipid raft content.

Moreover, direct cytotoxicity was observed after LiRecDT1 exposure by changes in morphology of RAEC cell cultures (Fig. 7A). Vacuolization of the cytoplasm, cell lysis debris, and alteration of cell–cell and cell–substrate adhesion were induced in a time-dependent manner by LiRecDT1 toxin treatment. The response of RAEC cells to LiRecDT1 H12A was very similar to untreated cells (negative control). The direct cell membrane interaction with dermonecrotic toxins does not presuppose cytotoxic effects, as this only occurs when significant enzymatic activity is present. Likewise, morphological changes in MDCK cells and proteinuria and renal tissue damage in mice were only triggered by recombinant phospholipase-D with intact enzymatic activity [28].

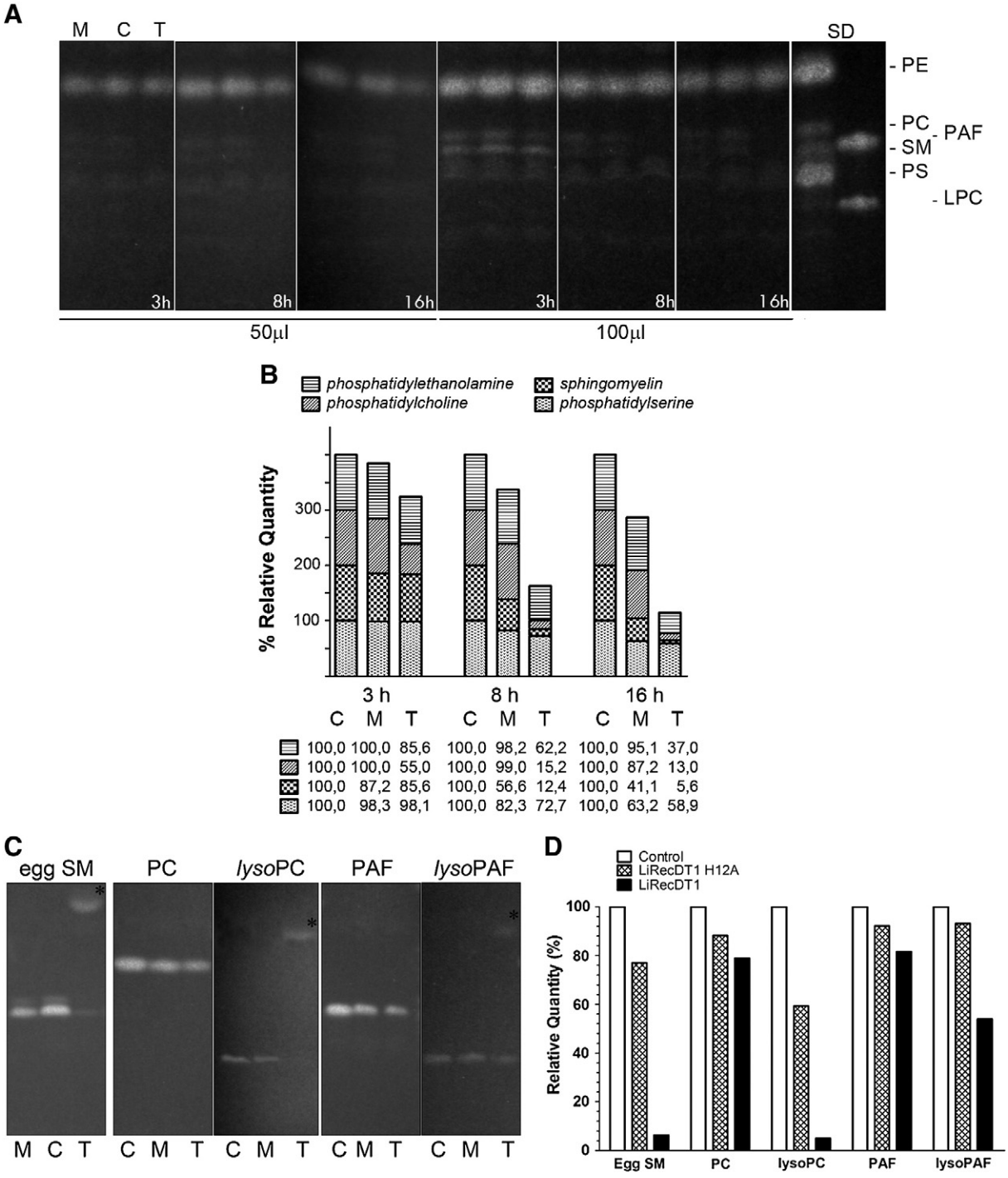

Fig. 8. HPTLC analysis of hydrolysis of RAEC cell membrane extracts and purified phospholipids by recombinant toxins. Triton X-100 extracts from endothelial cell cultures were incubated with 50 µg of either LiRecDT1 (T) or its mutated isoform, LiRecDT1 H12A (M), and non-treated extracts were used as control (C), for 3, 8, and 16 h. (A) Recovered lipids post-butanol extraction were visualized by primuline reagent in HPTLC plates, as described in Materials and methods. (C) After 3 h of incubation with toxins, purified lipids were also submitted to HPLC; egg chicken sphingomyelin (egg SM), asymmetric phosphatidylcholine C16:0–18:0 (PC), lysophosphatidylcholine (lysoPC), C16-2:0 PC (PAF), C16 Lyso PAF (lysoPAF). (B and D) Band intensities were estimated densitometrically and were plotted as percentage of relative quantity, comparing with respective control samples (100%). Percentage values are shown in the table (B), HPTLC bands of cell extracts lipid content were compared with migration patterns of phospholipids in the standard mixture (PE: phosphatidylethanolamine, PC: phosphatidylcholine, SM: sphingomyelin, PS: phosphatidylserine).

Phospholipase-D hydrolysis of the phosphodiester bond of glycerophospholipids generates (*lyso*)phosphatidic acid, and the degradation of sphingolipids forms ceramide. This reaction frees a headgroup, such as choline (resulting, for example, from sphingomyelin and (*lyso*)phosphatidylcholine [10,50]), which can act as a substrate for brown spider phospholipase-D. Choline release assays demonstrated direct hydrolysis of membrane extracts in a time- and concentration-dependent manner only with LiRecDT1 treatment (Fig. 8B), supporting the idea of catalytic-dependent cytotoxicity and suggesting that products of phospholipases-D degradation induce formation of bioactive mediators such as C1P or LPA [22,23].

In Fig. 8 (see *), the formation of subproducts induced by hydrolysis can be observed in HPTLC images, mainly in the top of those lanes where samples were exposed to active phospholipase-D and original lipid

content was almost degraded. Many lipids or lipid-derived products generated by phospholipases acting on phospholipids in membranes are implicated as mediators and second messengers in signal transduction [51]. For example, autotaxin (ATX) is an exo-enzyme originally identified as a tumor cell autocrine and paracrine motility factor. ATX is a multifunctional phosphodiesterase that primarily acts as a lysophospholipase D [52,53], converting lysophosphatidylcholine into LPA. LPA mediates multiple biological functions, including the cytoskeletal reorganization and chemotaxis observed in melanoma cells. Likewise, endothelial cell plasma membranes could be targeted by phospholipase-D activity, for which the regulation of lipid-derived second messenger levels intra- and intercellularly is not well understood [54]. Morphological alterations induced by phospholipase-D activity in RAEC cells could be explained by this hypothesis (Fig. 8B). Furthermore, the hallmark of brown spider dermonecrotic toxins is both dermonecrosis and hydrolysis of sphingomyelin, which in tissue context has been shown to be related to an inflammatory overreaction. This event is triggered by intense an neutrophil migration, which is dependent on the activation of fibroblast and endothelial cells by generating lipid mediators (such as C1P, an interconvertible molecule [22]) that cause a cascade of signaling events as pro-inflammatory cytokines upregulation and leukocytes recruitment [25].

As expected, LiRecDT1 and recombinant toxins obtained from *Loxosceles* genus venom glands have the ability to hydrolyze sphingomyelin and *lyso*phospholipids [10,55]. Interestingly, *L. intermedia* toxins also hydrolyze phospholipids such as phosphatidylcholine or PAF (platelet-activating factor), which are non alkyl-phospholipids (Fig. 8). The resulting lipid metabolite is phosphatic acid (PA), another bioactive metabolite related to LPA that has already been reported to play a role in stimulating several signaling pathways [56]. A juxtacrine mechanism for neutrophil adhesion on platelets involves platelet-activating factor and a selectin-dependent activation process, which are critical in cell–cell interactions in inflammatory and thrombotic responses of endothelial cells [54,57,58]. In the same way, hemostatic disturbances such as blood coagulation problems have been reported in with some brown spider bites [59]. In addition, platelet hyperaggregation was experimentally observed in human blood platelets exposed to a dermonecrotic toxin from *L. recluse* [60] and also thrombus formation and hemorrhage in rabbits tested with *L. gaucho* venom [61]. All phospholipids tested as substrates in HPTLC analysis with LiRecDT1 are important components/targets of plasma membranes of erythrocytes and platelets, for which phospholipase-D activity could be triggering several well known biological effects in brown spider accidents.

5. Conclusion

We present experimental data and literature supporting the direct involvement of dermonecrotic toxin in the biological effects evoked by brown spider venom. These data demonstrate phospholipase-D enzymatic activity on the endothelial cell membrane, suggesting a non-specific formation of different lipid mediators from cell membrane phospholipids. Our results bring new insights to the pathologic processes associated with a lipid role in pathways of the inflammatory response and dermonecrosis in Loxoscelism.

Acknowledgments

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