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Tyr → Trp-substituted peptide 115-129 of a Lys49 phospholipase A_2 expresses enhanced membrane-damaging activities and reproduces its in vivo myotoxic effect

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Abstract

Myotoxin II is a group II Lys49 phospholipase A_2 (PLA_2) isolated from the venom of the snake *Bothrops asper*. Previous studies on a synthetic peptide derived from its heparin-binding, cationic/hydrophobic sequence 115–129 demonstrated a direct functional role of this particular region in the in vitro cytolytic and bactericidal actions of the protein. Nevertheless, no significant myonecrosis has been observed after local intramuscular injection of peptide 115-129 (p115-129) in mice. Since the membrane-damaging action of p115-129 was proposed to depend on its amphiphilic character, the present study examined the effects of substituting its cluster of three tyrosine residues by tryptophan residues, on its toxic/pharmacological activities in vitro and in vivo. This substitution resulted in a drastic enhancement of the membrane-damaging activities of the peptide (p115-W3), together with the clear expression of myotoxic activity in vivo. Both the heparin-binding and antigenic characteristics of p115-129 were essentially conserved in p115-W3, suggesting that the modification did not lead to radical structural alterations. In addition to myotoxicity, cytotoxicity, and bactericidal action, p115-W3 exerted edema-forming activity in the mouse footpad assay. Thus, the synthetic 13-mer p115-W3 reproduced all the known toxic effects of myotoxin II. In spite of its potent membrane-damaging actions, p115-W3 did not acquire direct hemolytic activity upon mouse erythrocytes, an effect which is not present in myotoxin II, but that has been ascribed to the presence of tryptophan in other cationic, membrane-damaging peptides such as mellitin from bee venom. The myotoxic activity of p115-W3 herein described constitutes the first example of a short, PLA_2-based linear synthetic peptide with the ability to reproduce this effect of a parent protein in vivo. This finding is in clear support of the proposed relevance of the C-terminal region 115–129 in all the membrane-damaging mechanisms exerted by myotoxin II, including the myotoxic mechanism.

Keywords: Phospholipase A_2; Synthetic peptide; Myotoxin; Bactericidal; Snake venom

1. Introduction

Phospholipases A_2 (PLA_2s; EC 3.1.1.4) are abundant components of snake venom secretions that

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may display a diversity of toxic/pharmacological actions [1]. Among these stands myotoxicity, which contributes to the digestion of the muscle mass of the snake's preys, or causes significant skeletal muscle necrosis in accidentally envenomed humans [2]. Myotoxic PLA_2s from snake venoms have been classified as group I (from elapids) or group II (from viperids/crotalids) on the basis of their primary structure [3]. It was recently suggested that acquisition of myotoxic activity by these two groups of PLA_2s might have been a convergent evolutive process [4]. The molecular basis of the myotoxic activity of PLA_2s is still unclear. Despite the common morphological pattern of muscle necrosis induced by group I and group II myotoxic PLA_2s (see reviews in [2,5,6]), in vitro studies have provided evidence for the existence of distinct mechanisms of action. For example, while in vitro cytolytic activity is a common characteristic of all group II myotoxic PLA_2s tested to date, it was shown that notexin, a potent group I myotoxic PLA_2 from *Notechis scutatus scutatus*, completely lacks cytolytic effect under identical conditions [7,8]. Therefore, structure-function considerations concerning myotoxic PLA_2s cannot be extrapolated across group I and group II proteins.

The group II PLA_2 myotoxins have been further divided into two general categories: Asp49 and Lys49 variants. The latter proteins are considered PLA_2 homologues, since they lack (or may possess extremely low) catalytic activity on conventional substrates [9,10]. Nevertheless, they exert myotoxic and membrane-damaging actions comparable to the former [11–14], providing a useful model to study the catalytic-independent mechanism of muscle damage and to identify the key structural determinants of this activity.

Myotoxin II is a highly basic, dimeric Lys49 PLA_2 isolated from the venom of *Bothrops asper* [12,15,16]. The identification of structural determinants of its toxic action is being pursued (reviewed in [6,17]). A heparin-binding, cationic/hydrophobic region comprising residues 115–129 (numbering according to [18]) of this protein was demonstrated to be involved in its cytolytic mechanism in vitro: its corresponding 13-mer synthetic peptide 115-129 lysed endothelial cells, albeit with a markedly lower efficiency than the protein [19]. Subsequent work reported the bactericidal activity of both myotoxin II and its peptide 115-129, revealing the first catalytic-independent bactericidal mechanism for a secreted PLA_2 [20]. The relevance of region 115–129 in the muscle-damaging action of myotoxin II has been supported by demonstrating that antibodies against peptide 115-129 reduce the myonecrosis induced by this protein [17,21]. Nevertheless, a direct myotoxic effect of peptide 115-129 in vivo has not been observed, suggesting that additional structural determinants of the protein might be required for the full expression of myotoxicity [17,22].

The damaging action of peptide 115-129 on membranes was proposed to depend on its amphiphilic character [19]. This 13-mer peptide displays a prominent hydrophobic cluster containing three tyrosines, surrounded by several positively charged lysine/arginine side chains [15]. Consequently, while the cationic residues might establish electrostatic interactions with (yet unidentified) membrane constituents, the hydrophobic residues might disturb phospholipid bilayer integrity, initiating a rapid cell death process [17]. In order to test this model, it was hypothesized that substitution of the tyrosine residues by the more hydrophobic amino acid tryptophan (hydropathy indexes of -1.3 and -0.9, respectively [23]), without altering the charge pattern, might influence the membrane-damaging action of this peptide. The aim of this study was to examine the consequences of the tyrosine to tryptophan substitution on the in vitro and in vivo toxic/pharmacological activities of peptide 115-129 from myotoxin II.

2. Materials and methods

2.1. Synthetic peptides

Two 13-mer peptides, with native endings, were synthesized using Fmoc strategy [24]. Peptide p115-129 (numbering system of [18]) corresponds to residues 105–117 (KKYRYYLKPLCKK) of myotoxin II [15]. Peptide p115-W3 corresponds to a triple tyrosine to tryptophan substitution of the former (KKWRWWLKPLCKK). Purity of the peptides was 94.2% and 98.5%, respectively, as assessed by RP-HPLC on a C-18 column, and mass spectrometry

analyses. The measured molecular masses of p115-129 and p115-W3 were 1730.8 and 1799.5 Da, respectively, corresponding to the expected values (1730 and 1799 Da). Peptides were dissolved at the required concentrations in 0.12 M NaCl, 40 mM sodium phosphate (PBS), pH 7.2, prior to determining their biological activities.

2.2. Immunochemical cross-reactivity

Cross-recognition of p115-W3 by antibodies against p115-129 was evaluated by enzyme immunoassay, as described [17]. Microplates were coated by incubating 0.4 μg of each peptide/100 μl/well in 0.1 M Tris, 0.15 M NaCl, pH 9.0, overnight at room temperature. The binding of rabbit anti-p115-129 was detected with an alkaline phosphatase conjugate of anti-rabbit immunoglobulins (Sigma) and *p*-nitrophenyl phosphate. Normal rabbit serum was assayed in parallel against each peptide, as a control. Absorbances were recorded on a Dynatech MR5000 reader.

2.3. Heparin-binding activity

Heparin with low affinity for antithrombin [25] was added in gradual increments to a cuvette containing 200 μg of p115-W3 (approx. 55 μM), in 2 ml of 0.05 M Tris-HCl, 0.01 KCl, pH 7.0 buffer. Absorbance changes due to turbidity were monitored at 340 nm, allowing 1 min of incubation after each heparin addition [19].

2.4. Myotoxic activity

Groups of four Swiss mice (18–20 g body weight) received an intramuscular injection of varying amounts of p115-W3, in 100 μl of PBS, in their right gastrocnemius. Control mice received an injection of 100 μl of PBS. At different time points, blood was collected from the tail into heparinized capillary tubes, and the plasma creatine kinase (CK; EC 2.7.3.2) activity was determined (kit No. 520, Sigma). CK activity was expressed in U/ml, 1 unit resulting in the phosphorylation of 1 nmole of creatine per min at 25°C. Injected muscle samples were obtained after 24 h, fixed in Karnovsky's solution and processed as described [26] for histological analysis.

2.5. Edema-forming activity

Groups of four mice (24–26 g) received a subcutaneous injection of either p115-W3 (25 μg) in 100 μl of PBS, or 100 μl of PBS alone, in their right footpad. Edema was estimated by the increase in thickness of the injected footpad, which was measured with a low-pressure spring caliper at different time intervals [27].

2.6. Cytotoxic activity

The cytolytic effect of p115-W3 was determined using the murine endothelial cell line tEnd as a target, under identical conditions as described previously for p115-129 [7,19]. Cells were grown in 96-well plates in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), 2 mM glutamine, 1 mM pyruvic acid, penicillin (100 U/ml), streptomycin (0.1 mg/ml), and amphotericin B (0.25 μg/ml), in a humidified atmosphere with 7% CO_2, at 37°C, until reaching near confluence. After aspirating their growth medium, different amounts of p115-W3 diluted in assay medium (DMEM with 1% FCS) were added to the cultures, in a volume of 150 μl/well. After 3 h at 37°C, lactic dehydrogenase (LDH) activity released from damaged cells to supernatants was determined (Sigma No. 500). Controls for 0% and 100% toxicity consisted of assay medium, and 0.1% Triton X-100 in assay medium, respectively [7].

2.7. Hemolytic activity

Washed mouse erythrocytes were prepared from citrated blood, and resuspended at approx. 2% (v/v) in PBS. Different amounts of p115-W3, in 50 μl, were added to 250 μl of the red cell suspension, and incubated at 37°C. After 30 min, 2 ml of PBS were added, and the release of hemoglobin was estimated by determining the absorbance of supernatants at 540 nm. PBS alone or 0.1% Triton X-100 were utilized, respectively, to establish the 0 and 100% reference values.

2.8. Bactericidal activity

Escherichia coli (ATCC 29648) was utilized as a

target for determining bactericidal activity, by incubating 4×10^5 cells with varying amounts of p115-W3, for 20 min at 37°C, in PBS containing 1% peptone. Surviving bacteria were counted by the dilution plate technique, as described [20].

2.9. Intravenous and intraperitoneal lethal activity

Groups of three mice (16–18 g) received either an intravenous or an intraperitoneal injection of p115-W3 (100 μg) in 0.2 ml of PBS. Deaths were recorded during the following 48 h.

3. Results

3.1. Antigenic and heparin-binding properties of p115-W3

Rabbit antibodies raised against p115-129 showed a significant cross-reactivity with p115-W3, as determined by the enzyme immunoassay titration curves (Fig. 1), resulting in a binding signal of 60–70% relative to the homologous antigen. On the other hand, the heparin-binding activity of p115-W3 in solution was evidenced by the rapid formation of insoluble complexes, which caused maximal turbidity at a heparin/peptide molar ratio of 0.10–0.15 (Fig. 2). Further additions of heparin gradually reversed the turbidity, showing the reversibility of complex formation at different heparin/peptide ratios, with resolubilization of complexes at heparin excess (Fig. 2).

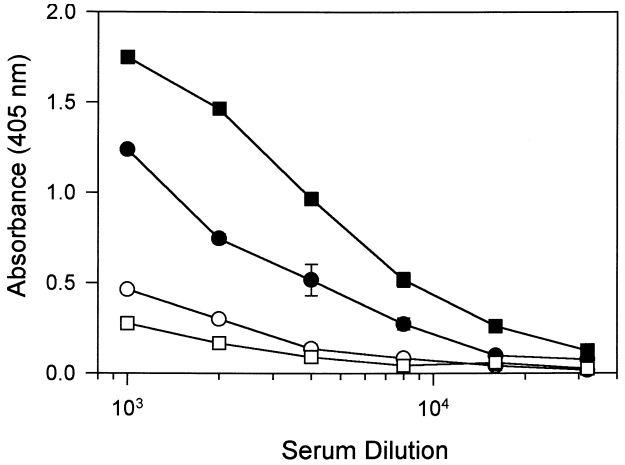

Fig. 1. Antibodies to peptide 115-129 cross-react with peptide 115-W3 by enzyme immunoassay. Different dilutions of rabbit antiserum to p115-129 (filled symbols) or normal rabbit serum (empty symbols) were added to microwells containing 0.4 μg of either p115-129 (■,□) or p115-W3 (●,○). Bound antibodies were detected by an anti-rabbit IgG/alkaline phosphatase conjugate, as described in Section 2. Each point represents the mean ± S.D. of triplicate wells.

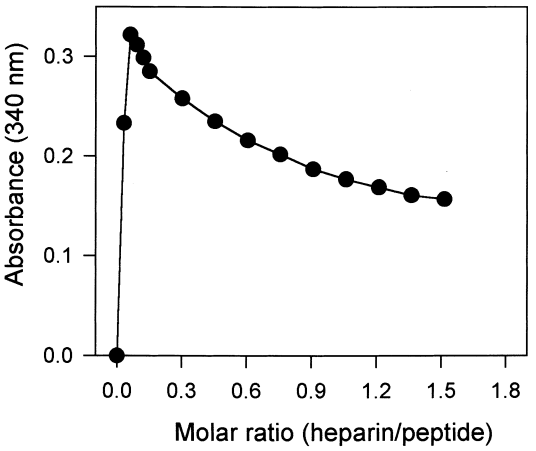

Fig. 2. Peptide 115-W3 forms macromolecular complexes with heparin in solution. Heparin was added in gradual increments to a cuvette containing 200 μg of p115-W3 (approx. 55 μM), in 2 ml of 0.05 M Tris-HCl, 0.01 KCl, pH 7.0 buffer. Turbidity was monitored at 340 nm, allowing 1 min of incubation after each heparin addition.

3.2. In vivo activities of p115-W3

Intramuscular injection of p115-W3 in mice resulted in a dose-dependent increase of plasma creatine kinase activity (Fig. 3A), which peaked by 1 h (Fig. 3B). Macroscopically, mice showed some difficulty in mobilizing the injected limb, which appeared moderately swollen, after a few minutes. Histological observation of injected muscle sections revealed areas containing numerous necrotic, hypercontracted myofibers with a clumped appearance (not shown). No signs of hemorrhage or other alterations were observed microscopically in the injected muscle tissue.

Subcutaneous injection of p115-W3 in the footpad of mice induced an immediate edema response, reaching a peak in the first 30 min, and gradually returning to basal levels by 6 h (Fig. 4).

Injection of up to 100 μg of p115-W3 (6 μg/g body weight) by either intravenous or intraperitoneal routes in mice did not result in death, or in any

evident alterations, during the following 48 h. Plasma creatine kinase levels after the intravenous or intraperitoneal injection of p115-W3 in these animals remained unaltered (data not shown).

3.3. In vitro activities of p115-W3

p115-W3 displayed cytolytic activity upon endothelial cells, in the concentration range of 30–50 μM (Fig. 5). This potency is approx. 15-fold higher than that reported for p115-129, under identical assay conditions [19]. The cytolytic effect of p115-W3 was completely neutralized when this peptide was pre-incubated with heparin at a 1:1 molar ratio (Fig. 5). On the other hand, p115-W3 did not cause lysis of mouse erythrocytes, over the same concentration range utilized in the cytotoxicity assay (data not shown).

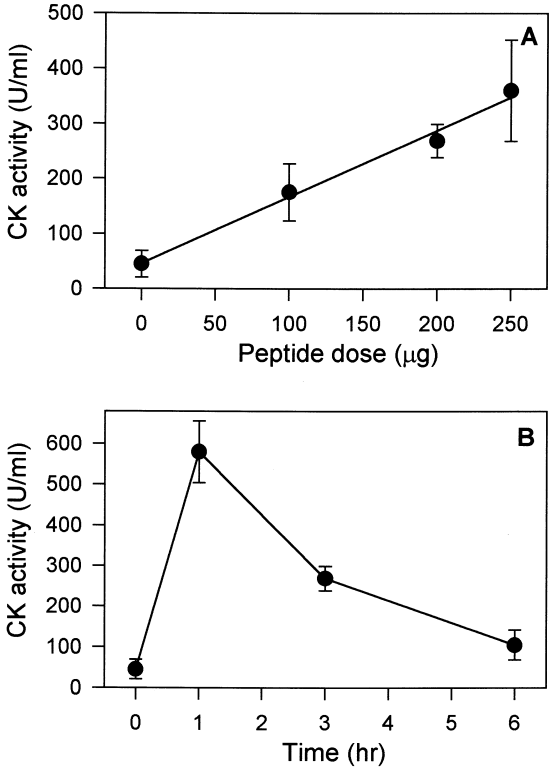

Fig. 3. Myotoxic activity of peptide 115-W3 in mice. (A) Different doses of p115-W3 were injected by intramuscular route in the right gastrocnemius of mice (18–20 g body weight) in a volume of 100 μl. Control mice received an injection of 100 μl of PBS. After 3 h, plasma creatine kinase (CK) activity was determined. (B) A fixed dose of p115-W3 (200 μg) was injected as described and the plasma CK activity was determined at the indicated time points. Each point represents the mean ± S.D. of four animals.

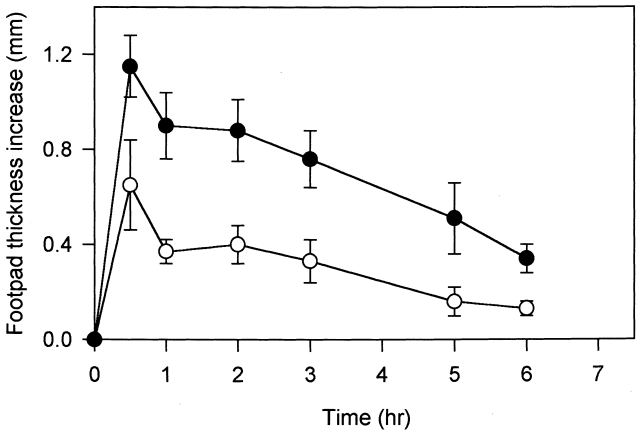

Fig. 4. Edema-forming activity of peptide 115-W3 in the mouse footpad assay. A fixed dose (25 μg) of p115-W3 in 100 μl of PBS (●) was injected subcutaneously in the footpads of mice (24–26 g body weight). Control mice received 100 μl of PBS alone (○). At the indicated time points, the increase in footpad thickness was determined as described in Section 2. Each point represents the mean ± S.D. of four animals.

Incubation of p115-W3 with *E. coli* 29648 at 37°C resulted in significant bacterial killing, in the

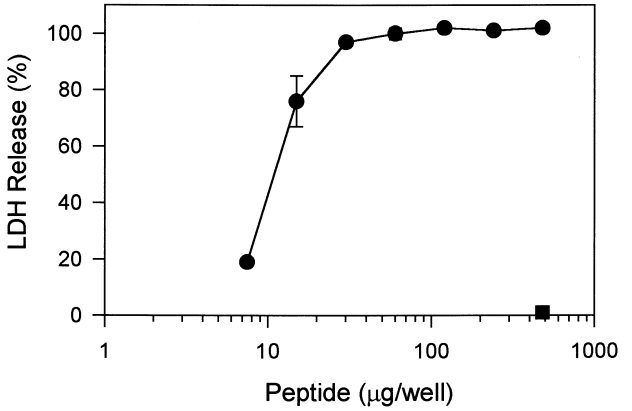

Fig. 5. Cytotoxic activity of peptide 115-W3 on murine endothelial cells in vitro, and its neutralization by heparin. Different amounts of p115-W3 (●) were added to tEnd endothelial cells growing in 96-well microplates, in a total of 150 μl (10 μg/well corresponding to approx. 35 μM of p115-W3). After 3 h of incubation at 37°C, cell culture supernatants were assayed for lactic dehydrogenase (LDH) activity, as an estimator of cytolysis. Medium alone or with 0.1% Triton X-100 was utilized as 0 and 100% reference values, respectively. Each point represents the mean ± S.D. of triplicates. To determine neutralization, cells were challenged with 480 μg of p115-W3 previously incubated for 15 min at 37°C with heparin (■), at a 1:1 molar ratio.

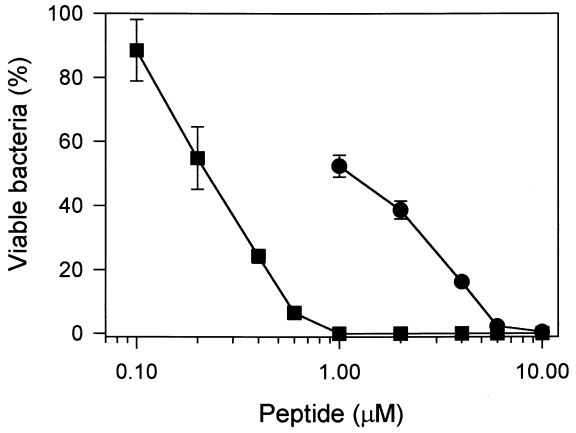

Fig. 6. Bactericidal activity of peptide 115-W3 on *E. coli*, compared to peptide 115-129. Varying amounts of p115-W3 (■) or p115-129 (●) were incubated with 4×10^5 *E. coli* (ATCC 29648) for 20 min at 37°C. Surviving bacteria were counted by the dilution plate technique. Each point represents the mean ± S.D. of four replicas.

0.1–1 μM range of concentrations (Fig. 6). The bactericidal activity of p115-W3 was at least 10-fold higher, in comparison to that of p115-129 (Fig. 6).

4. Discussion

The structural basis of the myotoxic action of Lys49 PLA_2s has begun to emerge in recent years. Neutralization studies on *B. asper* myotoxin II based on the use of heparin first highlighted the functional relevance of its region 115–129 [19]. Synthetic p115-129 reproduced the in vitro cytolytic [19] and bactericidal [20] actions of myotoxin II, although not its myotoxic activity in vivo (B. Lomonte, unpublished data). Since the action of peptide 115-129 on membranes is assumed to be dependent on its amphiphilic character [19], provided by the combination of cationic and hydrophobic residues, this study examined the effects of replacing the cluster of three tyrosine residues of p115-129 by tryptophan, a more hydrophobic amino acid. Results demonstrated that this substitution drastically enhances the membrane-damaging activities of the peptide. Both the antigenic and heparin-binding characteristics of p115-129 were essentially conserved in p115-W3, indirectly suggesting that the Tyr→Trp substitution did not lead to radical structural alterations, although this would have to be confirmed by direct analyses.

Interestingly, p115-W3 displayed a clear myotoxic activity in mice, as demonstrated by direct histological evaluation of injected muscle tissue, as well as by the dose-dependent increment in plasma creatine kinase activity. To the best of our knowledge, the myotoxic activity of p115-W3 herein described constitutes the first report of a PLA_2-based linear synthetic peptide with the ability to reproduce this effect of a parent protein in vivo. This finding is in clear support of the proposed role of region 115–129 as a determinant of the membrane-damaging mechanisms exerted by myotoxin II, including myotoxicity [6,17]. Other authors have suggested that, since the cationic/hydrophobic region 115–129 of Lys49 PLA_2s is not present in some neurotoxic/myotoxic Asp49 PLA_2s (i.e., crotoxin), it may not be essential for myotoxicity [28,29]. However, different types of PLA_2s may exert myotoxicity by diverse mechanisms [4,7,8], and major functional differences may exist even between the Asp49 and Lys49 PLA_2 variants [30]. In the case of the Lys49 PLA_2 myotoxin II, the present results agree with a key functional role of region 115–129 in myotoxicity, without excluding the participation of other protein determinants.

The observed enhancement of the membrane-damaging activities of p115-W3 demonstrates the important role of hydrophobic residues in its toxic mechanism. While maintaining the same positively charged residues of p115-129, the Tyr→Trp substitution of p115-W3 would favor hydrophobic interactions with phospholipid bilayers, leading to its increased toxic potencies. Tryptophan is known to be critically involved in the interaction of proteins with membranes [31,32]. Previous work reported the relevance of Trp110 in the lethal/neurotoxic action, but not in the enzymatic activity of the group I PLA_2 notexin [33]. The 115–129 region of a basic, myotoxic Asp49 PLA_2 from *Agkistrodon piscivorus piscivorus* [34] contains a natural Tyr→Trp substitution at position 119, compared to myotoxin II. In mellitin, a small cationic peptide from bee venom, it was demonstrated that the single Trp19 is essential for its membrane-damaging activity upon erythrocytes [35,36]. Noteworthily, in spite of its clear membrane-damaging activities, p115-W3 did not display hemolytic activity, even at concentrations causing complete cytolysis of cultured endothelial cells. In this regard p115-W3 resembled myotoxin II, which

lacks hemolytic activity, despite being cytolytic to a broad range of cell types [7]. Thus, while the Tyr→Trp substitution in p115-W3 drastically enhanced its membrane-damaging activities, it did not result in the acquisition of properties unrelated to those of its parent protein, such as hemolytic activity.

The edema-forming activity displayed by Asp49 PLA_2s has generally been attributed to their enzymatic activity [37,38], but several Lys49 PLA_2s can also induce this effect [12,39,40], probably by causing a direct degranulation of mast cells [39–41]. The edema-forming activity observed for p115-W3 is in agreement with the notion that Lys49 PLA_2s induce edema independently of an intrinsic catalytic activity. Moreover, results suggest that edema might be induced by the same structural motifs of the protein involved in myotoxicity, since p115-W3 was both myotoxic and edematogenic.

The basis for the selectivity of myotoxin II towards skeletal muscle fibers in vivo [6] as well as the nature of its membrane 'acceptor site' are still unknown. Since p115-W3 affected both eukaryotic membranes (causing myonecrosis in vivo or cytolysis in vitro), and prokaryotic membranes (being bactericidal to *E. coli*), it would be difficult to envisage that its myotoxic mechanism involves the interaction with specific proteic receptors for PLA_2s on skeletal muscle. This does not exclude the possibility that myotoxin II might utilize other protein region(s) to selectively target to such receptors, although no evidence along this line has yet been reported for the group II myotoxic PLA_2s. The general damaging activity of p115-W3 suggests that it interacts with essential membrane components which should be common to both eukaryotes and prokaryotes, possibly phospholipids or glycolipids. Previous studies, based on the construction of bacterial chimeras, demonstrated the interaction of myotoxin II and p115-129 with lipopolysaccharide moieties of Gram-negative bacteria [20].

The bactericidal action of p115-129 [20] was enhanced by at least one order of magnitude in p115-W3, which was effective in the 0.1–1 μM concentration range. In view of the small size (13-mer) of p115-W3 in comparison to other bactericidal cationic peptides, its efficient activity is worthy of notice as a possible lead compound for the development of novel antimicrobial agents.

In conclusion, the Tyr→Trp substitution of p115-129 markedly enhanced its membrane-damaging effects, demonstrating that it is possible to reproduce all the known toxic activities of *B. asper* myotoxin II, including its in vivo myotoxic effect, with only 13 amino acid residues. Indirectly, these findings stress the importance of region 115–129 as a major structural determinant of the toxic activities of myotoxin II, and possibly of related Lys49 PLA_2 myotoxins present in many crotalid snake venoms.

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References

[1] P. Rosenberg, in: W.T. Shier, D. Mebs (Eds.), Handbook of Toxicology, Marcel Dekker, New York, 1990, pp. 67–277.
[2] J.B. Harris, M.J. Cullen, Electron Microsc. Rev. 3 (1990) 183–211.
[3] E.A. Dennis, J. Biol. Chem. 269 (1994) 13057–13060.
[4] A. Alape-Girón, B. Persson, E. Cederlund, M. Flores-Díaz, J.M. Gutiérrez, M. Thelestam, T. Bergman, H. Jörnvall, Eur. J. Biochem. 259 (1999) 225–234.
[5] J.E. Fletcher, H.S. Selistre de Araujo, C.L. Ownby, in: R.M. Kini (Ed.), Venom Phospholipase A_2 Enzymes: Structure, Function, and Mechanism, John Wiley and Sons, England, 1997, pp. 455–497.
[6] J.M. Gutiérrez, B. Lomonte, in: R.M. Kini (Ed.), Venom Phospholipase A_2 Enzymes: Structure, Function, and Mechanism, John Wiley and Sons, England, 1997, pp. 321–352.
[7] B. Lomonte, A. Tarkowski, L.Å. Hanson, Toxicon 32 (1994) 1359–1369.
[8] B. Lomonte, Y. Angulo, S. Rufini, W. Cho, J.R. Giglio, M. Ohno, J.J. Daniele, P. Geoghegan, J.M. Gutiérrez, Toxicon 37 (1999) 145–158.
[9] C.J. Van den Bergh, A.J. Slotboom, H.M. Verheij, G.H. De Haas, J. Cell. Biochem. 39 (1989) 379–390.

[10] Y. Li, B. Yu, H. Zhu, M. Jain, M. Tsai, Biochemistry 33 (1994) 14714–14722.
[11] M.I. Homsi-Brandeburgo, L.S. Queiroz, H. Santo-Neto, L. Rodrigues-Simioni, J.R. Giglio, Toxicon 26 (1988) 615–627.
[12] B. Lomonte, J.M. Gutiérrez, Toxicon 27 (1989) 725–733.
[13] H. Kihara, R. Uchikawa, S. Hattori, M. Ohno, Biochem. Int. 28 (1992) 895–903.
[14] J.Z. Pedersen, B.F. De Arcuri, R. Morero, S. Rufini, Biochim. Biophys. Acta 1190 (1994) 177–180.
[15] B. Francis, J.M. Gutiérrez, B. Lomonte, I.I. Kaiser, Arch. Biochem. Biophys. 284 (1991) 352–359.
[16] R.K. Arni, R.J. Ward, J.M. Gutiérrez, A. Tulinsky, Acta Cryst. D51 (1995) 311–317.
[17] L. Calderón, B. Lomonte, Arch. Biochem. Biophys. 358 (1998) 343–350.
[18] R. Renetseder, S. Brunie, B.W. Dijkstra, J. Drenth, P.B. Sigler, J. Biol. Chem. 260 (1985) 11627–11634.
[19] B. Lomonte, E. Moreno, A. Tarkowski, L.Å. Hanson, M. Maccarana, J. Biol. Chem. 269 (1994) 29867–29873.
[20] L. Páramo, B. Lomonte, J. Pizarro-Cerdá, J.A. Bengoechea, J.P. Gorvel, E. Moreno, Eur. J. Biochem. 253 (1998) 452–461.
[21] L. Calderón, B. Lomonte, Toxicon 37 (1999) 683–687.
[22] C. Díaz, A. Alape, B. Lomonte, T. Olamendi, J.M. Gutiérrez, Arch. Biochem. Biophys. 312 (1994) 336–339.
[23] J. Kyte, R.F. Doolittle, J. Mol. Biol. 157 (1982) 105–132.
[24] R.M. Valerio, A.M. Bray, R.A. Campbell, A. DiPasquale, C. Margellis, S.J. Rodda, H.M. Geysen, N.J. Maeji, Int. J. Pept. Protein Res. 42 (1993) 1–9.
[25] B. Lomonte, A. Tarkowski, U. Bagge, L.Å. Hanson, Biochem. Pharmacol. 47 (1994) 1509–1518.
[26] L. Moreira, G. Borkow, M. Ovadia, J.M. Gutiérrez, Toxicon 32 (1994) 977–987.
[27] B. Lomonte, A. Tarkowski, L.Å. Hanson, Inflammation 17 (1993) 93–105.
[28] H.S. Selistre de Araujo, S.P. White, C.L. Ownby, Arch. Biochem. Biophys. 326 (1996) 21–30.
[29] S. Fujii, K. Ikeda, K. Hayashi, J. Toxicol. Toxin Rev. 17 (1998) 279–313.
[30] S. Rufini, P. Cesaroni, A. Desideri, R. Farias, F. Gubensek, J.M. Gutiérrez, P. Luly, R. Massoud, R. Morero, J.Z. Pedersen, Biochemistry 31 (1992) 12424–12430.
[31] W.M. Yau, W.C. Wimley, K. Gawrisch, S.H. White, Biochemistry 37 (1998) 14713–14718.
[32] S.F. Baker, R. Othman, D.C. Wilton, Biochemistry 37 (1998) 13203–13211.
[33] P. Mollier, S. Chwetzoff, F. Bouet, A.L. Harvey, A. Menez, Eur. J. Biochem. 185 (1989) 263–270.
[34] J.M. Maraganore, R.L. Heinrikson, J. Biol. Chem. 261 (1986) 4797–4804.
[35] S.E. Blondelle, R.A. Houghten, Pept. Res. 4 (1991) 12–18.
[36] S.E. Blondelle, L.R. Simpkins, E. Perez-Paya, R.A. Houghten, Biochim. Biophys. Acta 1202 (1993) 331–336.
[37] G. Cirino, S.H. Peers, J.L. Wallace, R.J. Flower, Eur. J. Pharmacol. 166 (1989) 505–510.
[38] S. Lloret, J.J. Moreno, Toxicon 31 (1993) 949–956.
[39] C.S. Liu, J.M. Chen, C.H. Chang, S.W. Chen, C.M. Teng, I.H. Tsai, Biochim. Biophys. Acta 1077 (1991) 362–370.
[40] E.C. Landucci, R.C. Castro, M.F. Pereira, A.C. Cintra, J.R. Giglio, S. Marangoni, B. Oliveira, G. Cirino, E. Antunes, G. De Nucci, Eur. J. Pharmacol. 343 (1998) 257–263.
[41] J.P. Wang, C.M. Teng, Eur. J. Pharmacol. 190 (1990) 347–354.