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Moving pieces in a cellular puzzle: a cryptic peptide from the scorpion toxin Ts14 activates AKT and ERK signaling and decreases cardiac myocyte contractility via dephosphorylation of phospholamban

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Abstract

Cryptic peptides (cryptides) are biologically active peptides formed after proteolysis of native precursors present in animal venoms, for example. Proteolysis is an overlooked post-translational modification (PTM) that increases venom complexity. The tripeptide KPP (Lys-Pro-Pro) is a peptide encrypted in the C-terminus of Ts14 – a 25-mer peptide from the venom of *Tityus serrulatus* scorpion that has a positive impact on the cardiovascular system, inducing vasodilation and reducing arterial blood pressure of hypertensive rats, among other beneficial effects. A previous study reported that KPP and its native peptide Ts14 act via activation of the bradykinin receptor B2 (B2R). However, the cellular events underlying the activation of B2R by KPP are unknown. To study the cell signaling triggered by the Ts14 cryptide KPP, we incubated cardiac myocytes isolated from C57BL/6 mice with KPP (10^{-7} mol.L^{-1}) for 0 min, 5 min or 30 min and explored the proteome and phosphoproteome. Our results showed that KPP regulated cardiomyocyte proteins associated with, but not limited to, apoptosis, muscle contraction, protein turnover, and the respiratory chain. We also reported that KPP led to AKT phosphorylation, activating AKT and its downstream target eNOS. We also observed that KPP led to dephosphorylation of phospholamban (PLN) at its activation sites (S16 and T17) leading to reduced contractility of treated cardiomyocytes. Some cellular targets reported here for KPP (e.g., AKT, PLN and ERK) have already been reported to protect cardiac tissue of hypoxia-induced injury. Hence, this study suggests potential beneficial effects of this scorpion cryptide that needs to be further investigated, for example as a drug lead for cardiac infarction.

**Keywords:** Apoptosis, Cryptide, Cardiomyocytes, Cardioprotection, *Tityus serrulatus*, Phosphoproteomics, Scorpion, Venome
Introduction

Arthropod venoms are natural sources of a wide spectrum of biologically active molecules, including neurotoxic, haemolytic, antiarrhythmic, anticonvulsant, anti-tumoral, anti-inflammatory, antimicrobial, and insecticidal agents, among others.\(^1\)\(^-\)\(^7\) Targets of these molecules can be receptors, ion channels, enzymes or other proteins in cells that may play critical roles in homeostasis. Therefore, venom-derived molecules represent a prominent repository of drug-lead candidates.\(^5\)

Molecular complexity of venoms is in part originated due to protein post-translational modifications (PTMs). Proteolysis is an overlooked PTM in the field that generates a myriad of cryptic peptides (also known as cryptides) from native toxins.\(^8\)\(^-\)\(^10\) For example, a bradykinin-potentiating peptide (BPP) named Peptide T was identified in the venom of the yellow scorpion \(Tityus\ serrulatus\).\(^11\) Later it has been shown that Peptide T was a cryptide from the neurotoxin Ts3.\(^12\) Attempting to better understand how PTMs impact toxins variability from \(Tityus\ serrulatus\) venom, a study used bottom-up and top-down proteomics. Among phosphorylated and \(N\)-linked glycosylated toxins, the authors also reported proteolysis as an important PTM of this venom, being several cryptides from the Ts14 reported in the study.\(^13\) Ts14 is a 25-mer peptide that acts directly via bradykinin B2 receptor (B2R) to elicit vasodilation, hypotension and anti-hypertensive effects. Cardiovascular activities elicited by Ts14 are associated to the KPP sequence located on its C-terminus, and the \(\xi\)-NH\(^3\)+ group on lysine side chain was shown to be essential to activate B2R.\(^14\)\(^-\)\(^15\)

Although some biological activities of the cryptide KPP have been documented,\(^14\) we lack information on the molecular events downstream of KPP-induced B2R activation. Thus, we addressed a time-resolved proteome and phosphoproteome study of mice cardiomyocytes exposed to KPP for 5 min (T5) and 30 min (T30). An untreated group was used as a control group and referred as 0 min of treatment (T0). Our data indicate that KPP regulates proteins and phosphoproteins associated to cardiomyocytes apoptosis and contractility. We also report that KPP induced the phosphorylation of AKT and ERK1/2 at their regulatory sites, S473 and T202/Y204, respectively. However, it remains to be experimentally determined whether the observed negative regulation of KPP on the cardiac
contraction is associated with the observed dephosphorylation of the cardiac phospholamban (PLN) at its regulatory site T17.

**Experimental procedures**

**Reagents**

Trypsin was purchased from Promega. Poros Oligo R2/ R3 reversed-phase material were from PerSeptive Biosystems. TiO$_2$ beads were from GL Science. Reprosil-Pur C18-AQ resins 3 µm and 5 µm were purchased from Dr. Maisch GmbH. Protease and phosphatase inhibitor cocktails were from Roche. All other reagents were obtained from Sigma otherwise stated.

**Animals**

Male C57BL/6 mice with 8 to 10 weeks-old and (25-30 g) were purchased from the Central Animal Care Facilities and from the Physiology and Biophysics Department Bioterium (Federal University of Minas Gerais - UFMG). Animals were maintained at standard laboratory conditions with access to water and food *ad libitum*. All the experimental protocols were conducted in accordance to the NIH Guide for the Care and Use of Laboratory Animals and approved by the local ethical committee (CEUA – UFMG, Protocol No. 213/2016).

**KPP peptide synthesis**

Synthetic KPP tripeptide was generated using standard solution phase coupling conditions. In general, N-Boc protected amino acids (1.1 molar equivalents) were activated with hexafluorophosphate azabenzotriazole tetramethyl uronium (HATU, 1.1 eq.), 1-hydroxy-7-azabenzotriazole (HOAt, 1.1 eq.), and diisopropylethyl amine (DIPEA, 4.0 eq.) in minimal dimethylformamide (DMF) prior to addition to C-terminal methyl ester protected amino acids (1.0 eq.) in dichloromethane. Synthetic N- and C-terminally protected di- and tripeptide intermediates were purified by silica flash chromatography and validated by NMR and mass spectrometry. Following acidic and basic deprotection, KPP was purified using C18 RP-HPLC.
Cardiomyocyte isolation and KPP treatment

The cardiomyocyte isolation protocol was performed as previously described. Briefly, animals were euthanized, the heart was immediately removed and perfused using Langendorff apparatus using calcium free solution (NaCl 130 mmol.L\(^{-1}\); KCl 5.4 mmol.L\(^{-1}\); HEPES 25 mmol.L\(^{-1}\); MgCl\(_2\) 1 mmol.L\(^{-1}\); NaH\(_2\)PO\(_4\) 0.33 mmol.L\(^{-1}\); glucose 22 mmol.L\(^{-1}\) and insulin 100U.mL\(^{-1}\); pH 7.4). After that, the heart was perfused with 1.2 mg.mL\(^{-1}\) of type II collagenase (Worthington) and then minced into tissue pieces and filtered through a mesh. The isolated cardiomyocytes were then incubated with KPP 10\(^{-7}\) mol.L\(^{-1}\) at room temperature during 5 min (T5) and 30 min (T30). After each treatment time, cells were snap-frozen with liquid nitrogen. Untreated cardiomyocytes were used as control group (T0). The samples were stored at -80\(^\circ\)C until further use (Figure 1a).

Sample preparation for proteomics

Cardiomyocytes were prepared for proteomic analysis as described before. Briefly, cardiomyocytes were resuspended in the lysis buffer (urea 6 mol.L\(^{-1}\), thiourea 2 mol.L\(^{-1}\), TCEP 10 mmol.L\(^{-1}\), chloroacetamide 40 mmol.L\(^{-1}\), TEAB 50 mmol.L\(^{-1}\) and protease inhibitors) and incubated for 2 h at 25\(^\circ\)C. Samples were diluted 10-times and proteins were quantified using the Qubit assay (Thermo). Protein digestion was achieved with trypsin (1:50 enzyme/protein ratio) during 18 h at 25\(^\circ\)C. The trypsin reaction was quenched by addition of TFA 0.5% (v/v) (final concentration). Thereafter, samples were centrifuged and vacuum-dried prior to the dimethyl labeling protocol (Figure 1b).

On-column peptide labeling

Dimethyl labeling of tryptic peptides was performed according to the previous study. In summary, samples were solubilized in 5% (v/v) formic acid and loaded in Sep-PaK C-18 cartridges (Thermo). After washing with 0.6% (v/v) acetic acid, samples were labeled with light (T0), intermediate (T5) and heavy (T30) dimethyl labeling reagents and eluted with 500 µL of 0.6% (v/v) acetic acid and 80% (v/v) ACN. Labeling efficiency was verified on a MALDI TOF-TOF mass spectrometer (Bruker) before mixing the samples in a 1:1:1 ratio at the ICB-UFMG Proteomics Core Facility (LMProt). Four independent replicates (n = 4) for each time-point were prepared (Figure 1b).
**Phosphopeptide enrichment**

Phosphopeptide enrichment was conducted in accordance to the previous study\(^\text{20}\) with minor modifications. Briefly, 100 µg of labeled peptides was added in the loading buffer (80% (v/v) ACN, 5% (v/v) TFA, 1 mol.L\(^{-1}\) glycolic acid) and incubated for 15 min with approximately 3.6 mg of TiO\(_2\) beads under constant agitation at room temperature. After centrifugation, the supernatant containing unbound phosphopeptides was then incubated in the same conditions in a new tube containing 1.8 mg of equilibrated TiO\(_2\) beads. Phosphopeptides were eluted from TiO\(_2\) beads using 70 µL of 1.5% (v/v) NH\(_3\) and vacuum-dried (Figure 1b).

**Sample pre-fractionation**

For the proteome samples, labeled peptides were pre-fractionated as described before with minor modifications.\(^\text{21}\) Briefly, samples (~30 µg) were resuspended in solvent B (90% (v/v) acetonitrile and 0.1% (v/v) TFA), and loaded on TSKGel Amide 80 HILIC column (length, 15 cm; diameter, 2 mm; particle size, 3 µm) at a flow rate of 6 µL/min. The elution gradient consisted in 26 min 100-60% solvent B. Solvent A was TFA 0.1% (v/v). The collected fractions were combined into 10 fractions and vacuum-dried until further use.

For the phosphoproteome samples, labeled phosphopeptides were pre-fractionated using high pH reverse-phase LC using an UltiMate 300 LC system (Thermo). Phosphopeptides dissolved in solvent A (20 mM ammonium formate, adjusted to pH 9.6 by NH\(_4\)OH) were loaded onto an Acquity CSH C\(_{18}\) column (300 µm x 100 mm, 1.7 mm) and fractionated using a stepped gradient from 4-48% solvent B (80% acetonitrile and 20% 20 mM ammonium formate, adjusted to pH 9.6 with NH\(_4\)OH) with 2% steps height and 3 min step length, followed by 80% solvent B. Fractions were pooled into 4 fractions and vacuum-dried until further use.

**Mass spectrometry analysis**

Samples were dissolved in 0.1% (v/v) formic acid (solvent A). For proteomics, we used an EASY-nLC system (Thermo) coupled to a Q-exactive HF mass spectrometer (Thermo). Samples were loaded on a pre-column (3 cm length x 100 nm inner diameter, 5 µm Reprosil-Pur C18-AQ resin) and separated on an analytical column (20 cm x 75 nm inner diameter, 3 µm Reprosil-Pur C18-AQ resin)
using the following chromatographic gradient: i) 1–3% solvent B (95% (v/v) acetonitrile and 0.1% (v/v) formic acid) in 3 min; ii) 3–25% solvent B in 80 min; iii) 25–45% solvent B in 10 min; iv) 45–100% solvent B in 3 min at flow-rate 250 nL/min. MS instrument was operated in positive polarity and data-dependent acquisition (Top 20) mode. Eluting peptide ions were resolved (MS1 mass range \( m/z \) 400–1600) at 120000 FWHM at \( m/z \) 200 allowing accumulation of up to \( 3 \times 10^6 \) ions or 100 ms (MS AGC target settings). The 20 most intense peptide ions were selected (isolation window = \( m/z \) 1.2) for HCD fragmentation (28% normalized collision energy). MS2 AGC target settings were 100 ms (injection time) or \( 10^5 \) ions. The fragment ions were resolved with 15000 FWHM at \( m/z \) 200 and selected precursor ions were included in a dynamic exclusion list for 30s.

For phosphoproteomics, we used an UltiMate 3000 Nanoflow LC system coupled to an Orbitrap Fusion Lumos (Thermo). Chromatographic gradient was performed as follows: i) 2–26% solvent B (95% (v/v) acetonitrile and 0.1% (v/v) formic acid) in 75 min; ii) 26–45% solvent B in 15 min; iii) 45–95% solvent B in 7 min at flow-rate 285 nL.min\(^{-1}\). MS instrument was operated in positive polarity and data-dependent acquisition (top-speed) mode. Eluting peptide ions were resolved (MS\(^1\) mass range = \( m/z \) 375–1500) at 120000 FWHM at \( m/z \) 200 allowing accumulation of up to \( 4 \times 10^5 \) ions or 50 ms (MS1 AGC target settings). For MS2, ions were selected in the quadrupole (isolation window = \( m/z \) 1.4) for HCD fragmentation (30% normalized collision energy). MS2 AGC target settings were 35 ms (injection time) or \( 5 \times 10^4 \) ions. The fragment ions were resolved with 15000 FWHM at \( m/z \) 200 and selected precursor ions were included in a dynamic exclusion list for 20s.

Data analysis

Raw spectra were searched against the Uniprot \textit{Mus musculus} database (downloaded version July 2019, 16970 entries) using MaxQuant (v.1.5.5.2). Parameters included carbamidomethyl cysteine as fixed modification, methionine oxidation as variable modification and up to 2 missed cleavages for trypsin. Match between runs was enabled only for the proteome spectra. Proteins with FDR < 1% and containing at least 1 unique peptide were used in this study. For phosphoproteomic analyses, the phospho (STY) were selected as a variable modification (Figure 1b).
Data analyses were conducted in Perseus (log2 transform, normalization, phosphosite analysis), DanteR22 [histograms, QQ-plots, box plot, PCA and volcano plots and statistical analyses one-way ANOVA] (Supplemental Information) and Excel (phosphosite abundance normalization based on protein abundancy23). Proteins considered up-regulated when p-value < 0.05 and log-transformed ratio intensity (Log₂FC=Log₂B-Log₂A) was > 0.585; and down-regulated when p-value <0.05 and log-transformed ratio intensity < -0.585. Functional analyses of differentially regulated proteins were performed using ClueGO plug-in for Cytoscape v. 3.7.24,25 (Figure 1b).

Data availability
The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE26 partner repository with the dataset identifier PXD015068.

Western blot
The isolated cardiomyocytes were submitted to protein extraction using lysis solution (NaCl 100mmol.L⁻¹, Tris 50mmol.L⁻¹, EDTA-2Na 5mmol.L⁻¹, Na₄P₂O₇.10H₂O 50mmol.L⁻¹, MgCl₂ 1mmol.L⁻¹, pH 8.0) added with protease and phosphatase inhibitors. Extracted proteins were quantified and then separated on SDS-polyacrylamide gel electrophoresis (SDS-PAGE) at 120V for 2 h. Proteins were transferred to a PVDF membrane (BioRad) at 20V. Membranes were incubated during 20 h at 4°C with the primary antibodies (1:1000): Anti-Akt (sc-1619, Santa Cruz Biotechnology), Ser473 phosphorylated Anti-Akt (#4060, Cell Signaling), Anti-ERK (#9102L, Cell Signaling), Thr202/Tyr204 phosphorylated Anti-ERK (#4370S, Cell Signaling), Anti-PLN (sc-393930, Santa Cruz Biotechnology) and Thr17 phosphorylated Anti-PLN (sc-17024-R, Santa Cruz Biotechnology). Subsequently, membranes were incubated with the Anti-rabbit secondary antibody (A0545 - Sigma) for pAkt, ERK1/2, pERK1/2 and pPLN, Anti-goat (A5420 - Sigma) for Akt and Anti-mouse (A2554 – Sigma) for PLN. The protein bands were detected using ECL Plus® reagent (Thermo) and analyzed in ImageQuant TL® software using LAS4000 (GE HealthCare Life Science. Protein abundance was normalized calculating the phosphorylated antibody concentration/total antibody concentration (Figure 1c).
**Cardiomyocyte contractility**

Mouse cardiomyocytes were placed in a chamber with a glass coverslip base mounted on the stage of an inverted microscope (TCM 400). Cells were stimulated by using platinum bath electrodes with voltage pulses (40 V intensity during 5 ms, 1 Hz frequency). The cardiomyocytes were visualized on a PC monitor with a NTSC camera (MyoCamCCD100V; Ionoptix) and the images collected were used to measure cell shortening in response to electrical stimulation, using a video motion edge detection system (Ionoptix). The cell image was sampled at 240 Hz. Cell shortening was calculated from the output of the edge detector using an IonWizard A/D converter (Ionoptix) (Figure 1c).

**Results and Discussion**

**KPP modulates the proteome and phosphoproteome of mouse cardiomyocytes**

The quantitative proteomic analysis of cardiomyocytes following KPP incubation led us to identify and quantify a total of 2049 protein groups of which 77 were differentially regulated between T5 vs. T0 (20 up-regulated, 57 down-regulated), 114 proteins between T30 vs. T0 (32 up-regulated, 82 down-regulated), and 63 proteins between T30 vs. T5 (23 up-regulated, 40 down regulated) (Figure 2a and Supplemental Information Table S1).

Regarding the phosphoproteome data, we identified a total of 653 class I phosphopeptides (phosphorylation probability > 0.75); 135 of which were normalized by the respective protein intensity. Comparing T5 vs. T0 dataset, we observed 44 phosphopeptides (belonging to 31 different phosphoproteins) differentially regulated (29 up and 15 down regulated). In the T30 vs. T0 dataset, we obtained 49 phosphopeptides (belonging to 35 different phosphoproteins) differentially regulated (28 up-regulated and 21 down-regulated), and in the T30 vs. T5 dataset, we obtained 36 phosphopeptides (belonging to 30 distinct phosphoproteins) differentially regulated (17 up-regulated and 19 down-regulated) (Figure 2b and Supplemental Information Table S2).

The observed phosphoproteins regulated due to KPP treatment were phosphorylated preferentially at serine residues (~91%), followed by threonine
residues (~8%) and tyrosine residues (~1%), which is in accordance with a similar study (Supplemental Information Table S2). Predicted kinases involved in the observed phosphorylation events were CK2A1 (23%), CAMK2A (18%), TESK1 (18%), PRKACA (17%), ERK2 (12%), mTOR (6%) and PRKCD (6%).

Principal component analysis (PCA) suggests that KPP induced an early regulation of cardiomyocyte proteome (Figure 2c) and phosphoproteome (Figure 2d) as seen by the T5 and T30 datasets nicely separated from T0 dataset.

Functional analysis of the proteome dataset showed that most relevant pathways significantly enriched ($p < 0.05$) associated to KPP treatment included translation, apoptosis, respiratory electron transfer, and protein turnover, among others (Figure 3a). Enrichment terms for the phosphoproteome dataset indicated that apoptosis, cell-cell interaction and regulation of heart contraction process were the main pathways affected by KPP treatment (Figure 4a).

KPP regulates nuclear and cytoplasmic proteins and phosphoproteins related to apoptosis

Apoptosis and autophagy are catabolic processes that govern turnover of cells and cytoplasmic components by balancing cell survival and cell death factors. Nuclear elements that play important roles in programmed cell death and gene expression includes, but are not restricted to, Histone H1.1 (H1-1, ID: P43275), Histone H1.3 (Hist1h1d, ID: P43277), Histone H1.4 (Hist1h1e, ID: P43274) and Lamin-B1 (Lmnb1, ID: P14733). These nuclear proteins were differentially regulated by KPP at the proteome level (Figure 3b and Supplemental Information Table S1). These histone proteins belong to the H1 linker family and are involved in the stabilization of the nucleosome and chromatin relaxation to allow transcription. Besides, phosphorylation is the major PTM found in H1 histones and is essential for the regulation of gene expression during the cell cycle. In our study, we observed that 5 min of KPP treatment induced dephosphorylation of S2 in H1.4, and T4 in H1.5 (Hist1h1b). These dephosphorylation events were sustained in H1.4 until 30 min (Figure 4c and Supplemental Information Table S2). This result is in accordance with other studies reporting that induction of apoptosis leads to H1 histone dephosphorylation. Moreover, KPP induced phosphorylation of the apoptotic chromatin condensation inducer in the nucleus (Acin1, ID: Q9JIX8) at S729
after 30 min of treatment (Figure 4b-c). Acin, also known as Acinus, is a nuclear protein involved in the induction of apoptotic chromatin condensation after activation by CASP3.\textsuperscript{33} Hu et al.,\textsuperscript{34} showed that phosphorylation of acinus on S422 and S573 by nuclear AKT results in its resistance to caspase cleavage and inhibition of chromatin condensation, thus promoting cell-survival.

Still as a nuclear effect of KPP, Bcl-2-associated transcription factor 1 (Bclaf1, ID: Q8K0190) was phosphorylated at S395 (Supplemental Information Table S2). This protein was described initially as a regulator of apoptosis machinery due to its interaction with anti-apoptotic family members Bcl-2 and Bcl-xL. Bclaf1 has also been associated to ribonucleoproteins (RNPs) and proteins from the spliceosome complex.\textsuperscript{35, 36} Nonetheless, while Bclaf1 contains more than 30 sites probably phosphorylated by GSK-3β kinase, the phosphorylation effect on Bclaf1 remains unclear.\textsuperscript{37, 38}

At the cytoplasmic level, KPP seems to modulate apoptosis through events controlling protein synthesis and turnover such as the observed regulation of ribosomal proteins Rpl7a, Rps16, Rps7 and Rps9, and mitochondrial ribosomal proteins Mrpl10 and Mrpl12. These proteins were down-regulated following KPP treatment (Figure 3c and Supplemental Information Table S1). We can also point out the up-regulation of proteasome subunits Psme2, Psmb5, Psmb7, and ubiquitin C-terminal hydrolase L3 (Uchl3) (Figure 3c and Supplemental Information Table S1). Observed up-regulation of proteins from the proteasome complex together with down-regulation of ribosomal proteins may explain why KPP induced more down-regulation events in the proteome level (Figure 2a). One could argue that finding more down-regulated than up-regulated proteins is due to technical bias rather than reflecting the KPP treatment. However, as shown in the Supplemental Information (Figure S1 and Figure S2), the proteome data have been successfully normalized and the features are equally distributed.

We observed an increased phosphorylation state of Cofilin-1 (Cfl1, ID: P18760), an actin-binding protein crucial for depolymerization of actin cytoskeleton\textsuperscript{39, 40} (Supplemental Information Table S2). The LIM-kinase 1 phosphorylates cofilin-1 at S3, inhibiting this protein and causing the accumulation of actin filaments leading to decrease cell migration and increased cell apoptosis.\textsuperscript{41, 42} Another upstream element
of this pathway is the serine/threonine-protein kinase MRCK beta (Cdc42bpb, ID: Q7TT50), which we found phosphorylated at S1692 after KPP treatment (Supplemental Information Table S2).

**KPP affects proteins and phosphoproteins associated to mitochondrial activity**

KPP treatment seems to reduce mitochondrial activity due to the down-regulation of components of the electron transport chain - NADH dehydrogenase [ubiquinone] flavoprotein 1 mitochondrial NADH dehydrogenase (Ndufv1, ID: Q91YT0), NADH-ubiquinone oxidoreductase chain 1 (Mtnd1, ID: P03888), NADH-ubiquinone oxidoreductase chain 4 (Mtnd4, ID: P03911) and NADH-ubiquinone oxidoreductase chain 5 (Mtnd5, ID: P03921) after 30 min treatment (Figure 3 and Supplemental Information Table S1). Mitochondrial dysfunction could initiate an oxidative stress that promotes cell death.\(^{43}\) Moreover, the glutathione s-transferase kappa 1 (Gstk1, ID: Q9DCM2) and glutathione S-transferase P 1 (Gstp1, ID: P19157), belonging to the glutathione S-transferases (GST's) superfamily and directly involved in cell detoxification,\(^{44}\) were down-regulated by KPP (Supplemental Information, Table S1). Even though we observed down-regulation of important mitochondrial proteins, others were up-regulated by KPP as, for example, the citrate synthase, mitochondrial (Cs, ID: Q9CZU6), the NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 10, mitochondrial (Ndufa10, ID: Q99LC3), and the NADP-dependent malic enzyme (Me1, ID: P06801) (Figure 3 and Supplemental Information Table S1). Therefore, the impact of KPP in mitochondrial homeostasis is still to be determined.

**KPP activates AKT/eNOS pathway**

Previously our group demonstrated that KPP mediates NO production via activation of the bradykinin B2 receptor (B2R) in mice cardiomyocytes.\(^{14}\) Since AKT is an upstream kinase of the nitric oxide synthase (eNOS), we hypothesized that the reported NO production due to KPP was a downstream effect of AKT activation. Indeed, KPP induced an early (5 min) phosphorylation of AKT at its regulatory site (S473)\(^{45}\) and this phosphorylation event was sustained for up to 15 min (Figure 5a). Hence, we believe that the activation of AKT results in the observed transient NO production.\(^{14}\) Interestingly, activation of the AKT/eNOS/NO pathway is associated to
a beneficial outcome in cardiac infarction.\textsuperscript{46-49} To investigate further the potential cardioprotection effect of KPP, we decided to evaluate whether this cryptide would be able to phosphorylate ERK1/2 at the regulatory sites T202 and Y204 as this protein has been associated to cell survival in response to NO augment via stimulation of soluble guanylyl cyclase (sGC).\textsuperscript{50} As shown in the Figure 5b, KPP induced the phosphorylation of ERK1/2 after 5 minutes of treatment.

**KPP decreases cardiomyocyte contractility**

Phospholamban (PLN, ID: P61014) is an important regulator of Ca\textsuperscript{2+} cycling and cardiac contractility. PLN is phosphorylated by PKA and CaMKII at S16 and T17, respectively.\textsuperscript{51} Dephosphorylation of PLN at both sites inhibits the sarcoplasmonic reticulum Ca\textsuperscript{2+}ATPase (SERCA2a) and thus the rate of Ca\textsuperscript{2+} uptake to the sarcolemma, reducing the myocardial contractility.\textsuperscript{52} We found that KPP induced dephosphorylation of cardiac phospholamban at S16 and T17 (Figure 4c and Supplemental Information Table S2) suggesting that KPP reduces cardiac contractility. This finding was validated by western blot that showed a significant dephosphorylation of PLN due to KPP treatment (Figure 6a). On the other hand, we observed that KPP induced phosphorylation of proteins interacting with PLN such as myosin-binding protein C, cardiac-type (Mybpc3 ID: O70468). We identified Mybpc3 phosphorylation at four sites (S72, T272, S273 and S307) due to KPP treatment (Figure 4c and Supplemental Information Table S2). Mybpc3 is a multiple phosphorylated protein substrate for PKA that acts as a key regulator of myocardial contractility since it interacts with titin, myosin and actin in sarcomeric organization.\textsuperscript{53} The phosphorylation state of Mybpc3 seems to be related to the regulation of thick–thin filament interaction and even a modest variation in its phosphorylation state can regulate cardiac myofilament function.\textsuperscript{54} In contrast, decreased phosphorylation of Mybpc3 was observed in cardiac ischemia-reperfusion model and pathological cardiac hypertrophy.\textsuperscript{56, 57} Taken together, these data suggest that KPP has a negative inotropic impact on cardiomyocytes (reduced contractility). To validate this hypothesis, we measured the contractility of the myocytes. Indeed, our functional validation showed that KPP reduced cardiomyocyte fractional shortening after 5 min of KPP treatment (Figure 6b). KPP did not reduce the cardiomyocytes fractional shortening after 30 min of treatment (Figure 6b), even though at this treatment time PLN was still dephosphorylated at T17 (Figure 6a). This finding
suggests that KPP induces later mechanisms, yet to be determine, to modulate the cardiomyocytes contractility.

**Conclusion**

Proteolysis is an overlooked PTM in Toxinology. To the best of our knowledge this is the first study showing the effect of a cryptide originated by proteolysis of the well-known scorpion toxin Ts14 on cardiac cell signaling. Here we demonstrated that KPP induced up-regulation of proteins associated to apoptosis at nucleic and cytoplasmic levels. KPP also regulated proteins related to cellular stress, like mitochondrial energetic shift, protein turnover, and muscle contraction. Moreover, we showed that the reported NO increased production by KPP is via AKT activation. We also showed that KPP led to PLN dephosphorylation which is in accordance to the observed reduced cardiac myocyte contractility after 5 min of KPP exposure. The observed KPP-modulated proteins and phosphoproteins suggest a potential beneficial effect of this cryptide in ischemic cardiac injuries, but the effectiveness of KPP as a cardioprotective agent is still to be determined. Finally, our study provides the first building blocks to better understand the signaling pathways induced by the scorpion venom cryptide KPP.

**Supporting information**

The following supporting information is available free of charge at ACS website http://pubs.acs.org

- Figure S1. Box-plot of protein and phosphoprotein normalized intensity values of identified proteins in mouse cardiomyocytes
- Figure S2. Histograms displayed normal distribution of protein and phosphoprotein intensity values identified in mouse cardiomyocytes
- Figure S3. Representative photos taken from the Western blotting membranes
- Supporting excel files (.xlsx):
  - Table S1. Differentially regulated proteins identified in mouse cardiomyocyte proteome after KPP incubation
Table S2. Differentially regulated phosphopeptides identified in mouse cardiomyocyte phosphoproteome after KPP incubation

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Conflict of Interest: The authors declare no conflicts of interest in regards to this manuscript.

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Figure 1. Workflow of the experimental approaches used to access and quantify the mice cardiomyocytes proteome and phosphoproteome following KPP treatment. (A) Mice cardiac left ventricles were used to isolate the cardiomyocytes. (B) Proteomics and phosphoproteomics highlighting important steps as 3-plex dimethyl labeling and phosphopeptide enrichment using titanium dioxide (TiO2). Peptides eluted from TiO2 beads were used to assess the phosphoproteome while unbound peptides (collected supernatant during TiO2 loading and washing steps) were used to assess the proteome. (C) Molecular and functional validation conducted by western blotting and cardiomyocyte contractility assay, respectively.
Figure 2. Differentially regulated proteins and phosphoproteins in cardiomyocytes after KPP treatment. Volcano plots represent the log2 ratio intensities (x axis) versus the p-value (y axis) for the (A) proteome and (B) phosphoproteome. The p-values were calculated using ANOVA. Dotted red lines show the cut-offs used to find the regulated features (p = 0.05, and log2 fold-change ratio > 0.585 (up-regulated) and log2 fold-change ratio < -0.585 (down-regulated). Points to the right represent positive regulation whereas points to the left represent negative regulation, when compared to T0 (left and middle panels) or T5 (right panel). Principal component analysis is shown for (C) proteome and (D) phosphoproteome datasets. T0, untreated cells; T5 and T30, cells were treated for 5 minutes or 30 minutes with KPP, respectively.
Figure 3. Pathway enrichment analysis for cardiomyocyte proteome regulated by KPP. Protein-protein interaction network was made on STRING and visualized on Cytoscape v.3.7.2. (A) Functional analysis was conducted on ClueGO application using Mus musculus database and default parameters. Only significantly enriched terms were plotted. (B) Network of regulated proteins contained in highlighted terms in (A). Related terms are linked through lines and marked with the same color. (C) Log2 ratio of regulated proteins represented in the networks in (B). Asterisk (*) denotes p < 0.05 and -0.585 < log2 fold-change > 0.585.

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Figure 4. Pathway enrichment analysis for cardiomyocyte phosphoproteome regulated by KPP. Protein-protein interaction network was made on STRING and visualized on Cytoscape v.3.7.2. (A) Functional analysis was conducted on ClueGO application using Mus musculus database and default parameters. Only significantly (p < 0.05) enriched terms were plotted. (B) Network of regulated phosphoproteins contained in highlighted terms in (A). Related terms are linked through lines and marked with the same color. (C) Log2 ratio of regulated phosphosites of proteins represented in the networks in (B). Asterisk (*) denotes p < 0.05 and -0.585 < log2 fold-change > 0.585.

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Figure 5. KPP activates PI3K/AKT/eNOS and MAPK/ERK pathways. (A) AKT phosphorylation was evaluated by western blot using anti-(pSer473)-AKT (anti-pAKT). Total AKT was also quantified using anti-AKT. Representative western blot images are shown in the upper panels. Quantification was achieved using pAKT/AKT ratio and shown as arbitrary units (a.u.). (B) ERK1/2 phosphorylation was evaluated by western blot using anti-(pThr202 / pTyr204)-ERK (anti-pERK). Total ERK was also quantified using anti-ERK. Representative western blot images are shown in the upper panels. Quantification was achieved using pAKT/AKT ratio and shown as arbitrary units (a.u.). Data are shown as mean +/- SEM. One-way ANOVA corrected for Holm-Sidak multiple comparisons. Only p-values < 0.05 are shown.
Figure 6. KPP regulates cardiomyocyte contractility via dephosphorylation of phospholamban (PLN). A) PLN phosphorylation was evaluated by western blot using anti-(pThr17)-PLN (anti-pPLN). Total PLN was also quantified using anti-PLN. Representative western blot images are shown in the upper panels. Quantification was achieved using pPLN / PLN ratio and shown as arbitrary units (a.u.). Data are shown as mean +/- SEM. One-way ANOVA corrected for Holm-Sidak multiple comparisons. Only p-values < 0.05 are shown. B) Fractional shortening measurement in response to electrical stimulation. Cardiomyocytes were pre-treated with KPP for 5 min (T5) or 30 min (T30). Untreated cardiomyocytes were used as control (T0). Data are shown as mean +/- SEM. One-way ANOVA corrected for Dunnett’s multiple comparisons. Only p-value < 0.05 is shown.