RESEARCH ARTICLE

Recombinant Phα1β Toxin (CTK 01512-2) Attenuates Abdominal Hyperalgesia and Inflammatory Response Associated with Acute Pancreatitis Induced by Cerulein in Rats

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ABSTRACT

Background: The molecular mechanisms underlying the chronic pain associated with pancreatitis are poorly understood. Hypercalcemia is a risk factor and the role of cytosolic calcium is described as a modulator of pancreatitis. Blockade of Ca^{2+} signals may be useful as prophylactic treatment of pancreatitis. Agents that modulate the activity of the Voltage-Sensitive Calcium Channels (VSCCs) exhibit experimentally and clinically significant pain relief in visceral pain. Between these agents, the toxins Phα1β and ω-conotoxin MVIIA are effectively reducing the chronic pain in rodent models. **Patients and Methods:** AP was induced in rats that received 5 times hourly an intraperitoneal injection of cerulein (5 μg/kg in 100 μL of volume). Blood serum pancreatic enzymes and ROS analysis were monitored after 8 hours of the first injection cerulein. Behavioral testing of the nociceptive state of the abdominal area was included in the study. **Results:** Compared with control groups, rats receiving cerulein demonstrated an increase in withdrawal events after analgesimeter stimulation. The abdominal mechanical hyperalgesia was reduced after application of the VSCCs blockers ω-conotoxin MVIIA, Phαβ native and recombinant CTK-01512-2. The treatments reduced the cerulein-induced increase in ROS, amylase and lipase levels. Phα1β and CTK 01512-2 did not affect the horizontal and vertical exploration activities while ω-conotoxin MVIIA increased then. **Conclusion:** These results indicate that animals injected with cerulein-induced experimental pancreatitis expressed by visceral hyperalgesia, ROS, serum amylase and lipase characteristics of pancreatitis. The treatments of the animals with the VSCCs reduced the effects of pancreatitis.

INTRODUCTION

Visceral pain is the most common form of pain that often motivates patients to seek medical attention. Surveys point to Acute Pancreatitis (AP) as the most common gastrointestinal disease, which affects about 10 to 45 cases per 100,000 population per year in the United States.

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Keywords Cerulein, Rats, Acute pancreatitis, Nociceptive behavior, Calcium channel blockers, Pα1β, ω-conotoxin MVIIA, Amylase, Lipase, ROS

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This kind of pain is the most common type and is a challenge for medicine since it is often resistant to medical treatments [1].

Despite considerable advances in knowledge about the mechanisms underlying visceral pain and visceral hyperalgesia, there are no new effective therapies for this type of abdominal pain [2]. The pharmacological treatment of pancreatitis pain is still limited with respect to efficacy and side effects [3]. Hence, an essential goal of treatment for pancreatitisis to relieve the pain. The analgesic treatment is often inadequate as the pathophysiology behind disease as well as the mechanisms behind the accompanying pain is not yet fully understood [4]. The molecular mechanisms underlying the chronic pain associated with pancreatitis are poorly understood, but within recent years, animal experiments have suggested some devices that might be involved.

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Hypercalcemia is a risk factor for pancreatitis; consequently, the role of cytosolic calcium has been described as a critical modulator of the initiation and development of pancreatitis pain [5]. Blockade of Ca^{2+} signals and mainly RYR-Ca^{2+} may be useful as prophylactic treatment of pancreatitis [6].

Agents that modulate the activity of the Voltage-Sensitive Calcium Channels (VSCCs) exhibit experimentally and clinically significant pain relief in visceral pain [7]. So far, most of the drugs targeting VSCCs are peptide toxins isolated from venom animals. The synthetic peptide toxin ω-conotoxin MVIIA (ziconotide) is a potent and selective blocker of the N-type calcium channel [8] and can reduce pain but the therapeutic window is narrow with severe, dose-limiting side effects [9]. The venom of the armed spider *Phoneutria nigriventer* contains peptide toxins that are potent and selective ion channel blockers with great pharmaceutical potential [10]. PnTx3-6 toxin, patented as Phα1β, reversibly inhibits voltage-gated calcium channels types N>P/Q>R>L [11] and TRPA1 channels [12], producing marked analgesic effect dissociated from toxicity in relevant models of rodents pain [9]. Therefore, the aim is to study the peptides toxins VSCCs Phα1β, CTK 01512-2 and ω-conotoxin MVIIA, as inhibitors of noxious stimuli, in the pathogenesis of pancreatic pain in a rat model of induced by cerulein. This non-invasive model is amongst the most widely used models of pancreatitis pain.

MATERIALS AND METHODS

The native Phα1β toxin purified from the PhTx3 fraction of the *Phoneutria nigriventer* venom as described by Cordeiro, et al., 1993 [13]. The recombinant Phα1β toxin (CTK 01512-2) was synthesized by Giotto Biotech (www.giottobiotech.com). Both toxins have the same sequence of 55 amino acids (ACIPRGEICTDDCECCGCDNQCYCPPGSSLGIFKCSCAHAN KYFCNRKKEKCKKA). ω-conotoxin MVIIA purchased from Latoxan (Valence, France). The stock solutions of the toxins were prepared in PBS and stored in siliconized plastic tubes (Eppendorf). Cerulein obtained from Sigma-Aldrich (CA, USA) dissolved in PBS and stored in concentrations of 10 μg/mL. All solutions stored at -20°C until processed.

Animals and experimental design

Adult male Wistar rats (140 g-200 g) obtained from CEBIO-ICB/UFMG and were habituated to animal room temperature controlled (22°C ± 2°C), treated under light/dark cycle with free access to water and food. The experiments followed the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH publications No. 8023 revised 1978) and the current guidelines for laboratory animal care in research experiments [14].

The ethics committee for experimentation with living animals from the Institute for education and research-hospital Santa Casa, Belo Horizonte, authorized all the procedures (Protocol 0002/2016).

Acute pancreatitis was induced with some modifications from previous studies [15]. The rats fasted for 12 hours before AP induction. Animals received 5 times hourly an intraperitoneal injection of cerulein (5 μg/kg in 100 μL of volume) in the lower right quadrant. Injections were performed at 1-hour period intervals over the four hours (5 doses). The experimental design of the induction of Acute Pancreatitis (AP) is shown in **Figure 1**.

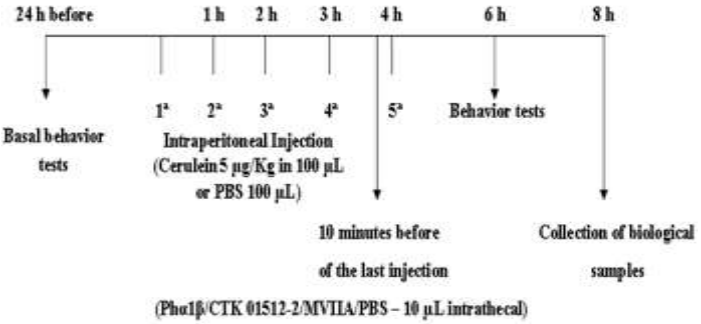

Figure 1: Design of the cerulein-induced of acute pancreatitis in rats.

Biological samples used to determination of biochemical and inflammatory parameters were collected after 8 hours of the first injection cerulein (maximum changes consistent with AP). Additionally, animals received intrathecal injections of Phα1β, CTK 01512-2, ω-conotoxon MVIIA or PBS (vehicle) 10 minutes before the last injection of cerulein. The behavioral evaluation was performed 2 hours after the last administration of cerulein (Figure 1). The rats were allocated to the following five experimental groups (n=8/group).

- Control group-animals that received, in all experimental conditions, only intraperitoneal injection of PBS (vehicle) and treated with the PBS.
- Cerulein group-animals submitted to the induction of AP by the injection of cerulein 5 μg/kg and treated with the PBS.
- Cerulein plus native Phα1β toxin group-animals subjected to induction of AP by the injection of cerulein 5 μg/kg and treated with the native Phα1β toxin (25 pmol/site-200 pmol/site).
- Cerulein plus recombinant CTK 01512-2 toxin group-animals subjected to induction of AP by the injection of cerulein 5 μg/kg and treatment with the recombinant CTK 01512-2 toxin (25 pmol/site to 200 pmol/site).

- Cerulein plus ω-MVIIA toxin group-animals subjected to induction of AP by the injection of cerulein 5 μg/kg treatment with the MVIIA toxin (10 pmol/site to 100 pmol/site).

The behavioral evaluation performed 2 hours after the last administration of cerulein (**Figure 1**).

Measurements of abdominal mechanical hyperalgesia

Using an analgesimeter (Ugo Basile, Italy) it was evaluated the mechanical hyperalgesia in the rat abdominal region as previously described by Camargo et al., 2011 [16]. The method allows an increasing force applied to the abdominal region of rats until any withdrawal behavior is observed when the threshold force value in the equipment is registered. Briefly, after the treatments, the animals were previously acclimatized for 1 hour in elevated plastic cages 12 cm × 20 cm × 17 cm, which allow the stimulation of the abdominal region of the animal through a mesh floor.

Quantification of the mechanical sensitivity of the abdominal region (lateral-anterior region) by measuring the force threshold (0.1 g-1000 g) required to cause the withdrawal response of the abdomen, whole body or licking of the abdominal region. The stimulus evaluation applied in triplicate at intervals of one minute between each one.

The values, for each animal, calculated as the average of three measurements with a difference of less than 10 grams. Data were expressed as the intensity of the stimulus threshold (in grams) required to the withdrawal response.

Serum amylase and lipase levels of determination

Serum amylase and lipase levels from each experimental group evaluated by the Zoogene-medical laboratory, Belo Horizonte, MG, Brazil using absorbance, photometry and turbidimetry techniques. Amylase evaluation by the caraway method 1959 [17], fixed time kinetics, in which the sample incubated with a starch substrate and by addition of iodine. The non-hydrolyzed starch acquired blue coloration decreased in proportion to the enzyme activity being compared with a control. Lipase levels in the samples using the colorimetric enzymatic method.

The chromogenic substrate of lipase 1, 2-O-dilauryl-rac-glicero-3-glutaric acid-(6-methylresorufin)-ester is cleaved by the catalytic action of the lipase alkaline solution forming 1, 2-O-dilauryl-rac-glycerol and an unstable intermediate, glutaric acid-(6-methylresorphine) ester. This acid spontaneously decomposes into alkaline solution forming glutaric acid and methylresorufin. The intensity of red color formed is directly proportional to lipase activity and is determined photometrically.

Free Radicals (ROS) content of the spinal cord

Segments of the spinal medulla (1 cm) from the L5-L6 (intrathecal region) were removed and transferred to 1.5 ml tubes. They were weighed and homogenized (1:10; w/v) in 50 mM phosphate buffer solution, containing 140 mM KCl at 4°C (pH 7.4).

The homogenate centrifuged at 1000 × g for 10 min at 4°C and the supernatant collected in 1.0 mL tubes and then frozen at -70°C for future biochemical evaluations.

The protein content measured according to the Bradford method (1976) [18] using bovine serum albumin as the standard (1 mg/ml). ROS measurements performed using 2', 7'-Dichlorofluorescein Diacetate (DCF-DA), fluorescent probe, as previously described by Siqueira, et al., 2004 [19] with modifications. Briefly, an aliquot of 20 μl of the sample added to 80 μl 125 μM DCF-DA stock solution at 37°C for 30 min and protected from light. Formation of the oxidized fluorescent derivative (DCF) was monitored at excitation and emission wavelengths of 488 and 525 nm, respectively, in a fluorescent plate reader (PerkinElmer, Waltham, MA, USA). The results were normalized by protein content.

Exploratory activity (Open field)

To assess the effect of i.t. of the Phα1β and CTK 01512-2 toxins on the spontaneous locomotor activity (horizontal and vertical exploration), the animals were placed on photocells measuring 40 cm × 12 cm × 40 cm. They were accustomed for 30 minutes and the activity was monitored for 60 minutes at 5-minute intervals. This activity was measured using an activity monitor using three infrared light detectors located on a photocell [20].

The horizontal exploration measured by the total distance traveled in centimeters (cm) and the vertical exploration by the time of rearings (in seconds) and of crossing (the number of horizontal detachments of the center of gravity of the animal).

Statistical analysis

Experimenters blinded to treatments collected data and expressed as mean ± standard error of the mean (mean ± S.E.M). Data were analyzed by one-way or two-way ANOVA followed by Bonferroni, Newman-Keuls, Dunn or Dunnet post-test when appropriate. Differences between groups were considered significant when $p<0.05$. Statistical analysis of the results was performed using graphpad prisma, version 5.0 for windows (GraphPad Software, La Jolla, CA, USA).

RESULTS

The evaluation of the nociceptive behavior (abdomen withdrawal threshold), as a function of the number of injections of intraperitoneal (ip) of cerulein (5 μg/Kg) is shown in **Figure 2**. There was no difference between the induced hypersensitivity by five or seven injections of cerulein i.p., $p>0.05$.

Thus, all the experiments for the action of the drugs on the hypersensitivity induced by cerulein in AP were performed using five injections of cerulein administered during 4 hours.

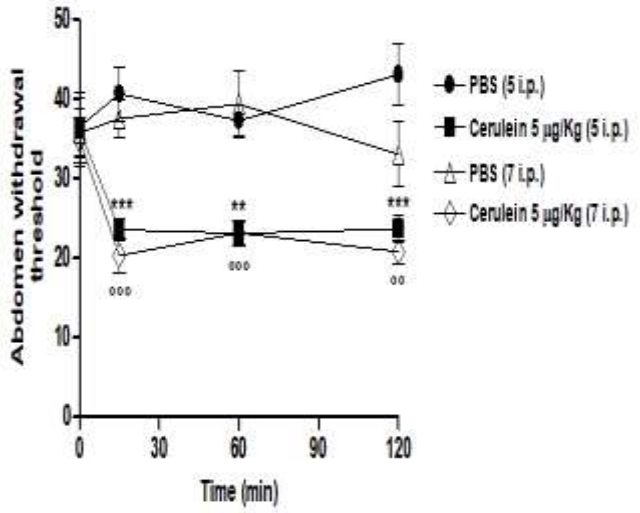

Figure 2: Nociceptive behavior as function of time (min) in two experimental conditions, 5 and 7 intraperitoneal injections of cerulean.

Note: Cerulein 5.0 µg/Kg in two experimental conditions of 5 and 7 of intraperitoneal injections in the induction abdominal nociceptive behavior of AP. n=8 per group. $^{**}p<0.01$ and $^{***}p<0.001$ in relation to the PBS group with 5 injections. $^{oo}p<0.01$ and $^{ooo}p<0.001$ in relation to the PBS group with 7 injections. Two-way ANOVA, Bonferroni post test.

The concentrations curve of the treatments with ω-conotoxin MVIIA, Phα1β and recombinant CTK-01512-2 on the mechanical hyperalgesia of AP-induced by cerulein (5 µg/kg, i.p) is shown on the Figures 3A, 3B and 3C. At the concentration of 100 pmol/site, ω-conotoxin MVIIA inhibited 74% ± 2.0%, of cerulein-induced hypersensitivity, p<0.01 with an ID50 de 51 ± 1.6 pmol/sitio.

There was no significant effect at the concentration of 10, 25 or 50 pmol/site, **Figure 3A**.

Phα1β reduced cerulein-induced mechanical hypersensitivity at the concentrations of 100 pmol/site or 200 pmol/site, p<0.001 and did not affect at the concentrations of 25 or 50 pmol/site, **Figure 3B**.

The ID50 for the Phα1β inhibition of the cerulein effect was 54 ± 2.5 pmol/site. Phα1β caused maximal inhibition at 100 pmol/site. Phα1β, recombinant (CTK-01512-2), 100 or 200 pmol/site reduced the abdominal hypersensitivity and the maximal inhibition was 100 ± 1.7%, p<0.001, respectively with an ID50 de 90 ± 1.2 pmol/site, **Figure 3C**.

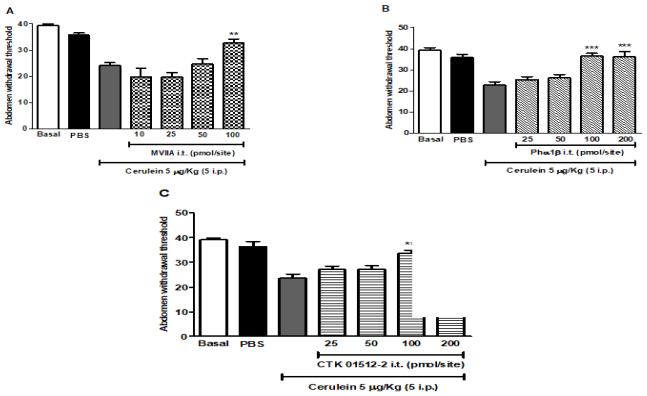

Figure 3: Dose-response curves of the abdominal mechanical hyperalgesia cerulein-induced acute pancreatitis in rats treated with Phα1β, CTK 01512-2 and ω-conotoxin MVIIA.

Note: Treatments with ω-conotoxin MVIIA. A: Phα1β; B: CTK 01512-2; C: by intrathecal route (n=8 per group); A: $^{**}p<0.01$ significance in relation to the cerulein group, ID50=51 pmol/site; B: $^{***}p<0.001$ significance in relation to the cerulein group; ID50=54 pmol/site; C: $^{**}p<0.01$; $^{***}p<0.001$ significance in relation to the cerulein group. ID50=90 pmol/site. One-way ANOVA, Newman-Keuls post test.

Cerulein (5 µg/kg, i.p,) induced increase of serum amylase and lipase of the rats with AP, p<0.001. The treatment of the rats with ω-conotoxin MVIIA (100 pmol/site i.t.) did not affect the levels of serum amylase and lipase. The treatments with Phα1β or with CTK 01512-2 (100 pmol/site i.t) reduced the increase on amylase levels of cerulein-induced by 89.70% ± 8.8% and 83.40% ± 8.5%, respectively (**Figure 4**). The reduction on the lipase by Phα1β and its recombinant CTK-01512-2 (100 pmol/site i.t) was about 94.3% ± 5.4% and 69.4% ± 13.0%, respectively, p<0.001.

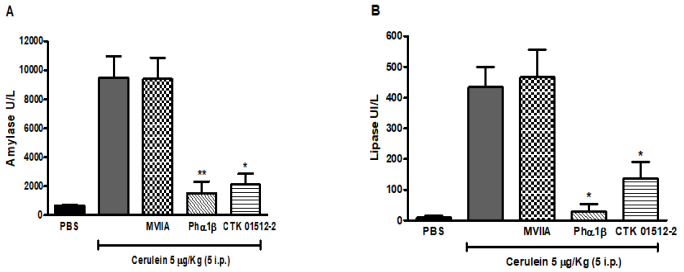

Figure 4: Effect of the toxins Phα1β, CTK 01512-2 and ω-conotoxin MVIIA, intrathecal route, in the increase of the cerulein-induced serum levels of the amylase and lipase during acute pancreatitis.

Note: Treatments with ω-conotoxin MVIIA, Phα1β, CTK 01512-2 (100 pmol/site) by intrathecal route (n=8 per group). A: $^{**}p<0.01$ significance in relation to the cerulein group; $^{*}p<0.5$ significance in relation to the cerulein group; one-way ANOVA, Dunn's multiple comparisons test. Amylase inhibition: 89.70% ± 8.8% by Phα1β and 83.40% ± 8.5% by CTK 01512-2. Lipase inhibition 94.3% ± 5.4% by Phα1β and 69.4% ± 13.0% CTK 01512-2. B: $^{*}p<0.5$ significance in relation to the cerulein group; one-way ANOVA, Dunn's multiple comparisons test.

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The increase on the ROS levels induced by cerulein 5 µg/kg, i.p administered rats with AP was the double of the PBS (control) from 409 ± 25 a.u to 865 ± 28 a.u., p<0.001, **Figure 5**. Treatments of the rats with AP with ω-conotoxin MVIIA (100 pmol/site) or CTK 01512-2 (100 pmol/site) i.t. reduced the cerulein-induced increase in ROS by 52.4% ± 13.2% and 65.1% ± 12.3%.

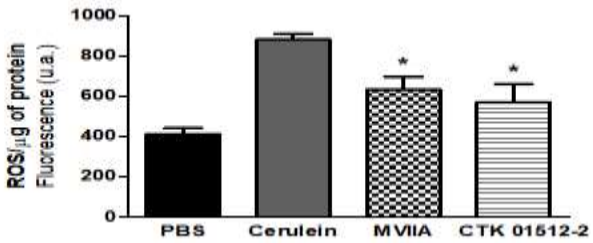

Figure 5: Effect of toxins ω-conotoxin MVIIA and CTK 01512-2, intrathecal route, in the production ROS during cerulein-induced acute pancreatitis.

Note: Treatments with ω-conotoxin MVIIA (100 pmol/site) and CTK 01512-2 (100 pmol/site) by intrathecal route (n=8 per group). *p<0.05 significance in relation to the cerulein group. One-way ANOVA, Newman-Keuls post test.

Figure 6 shows the results of the horizontal (6A) and vertical exploration (6B) of the rats with AP induced by cerulein, 2 h after the last administration of cerulein 5 µg/Kg (i.p). There was no effect of cerulein in the horizontal and vertical exploration. Treatment of cerulein-induced AP with ω-conotoxin MVIIA (100 pmol/site i.t) increased the horizontal and vertical exploration while Phα1β and CTK 01512-2 did not affect these exploratory activities.

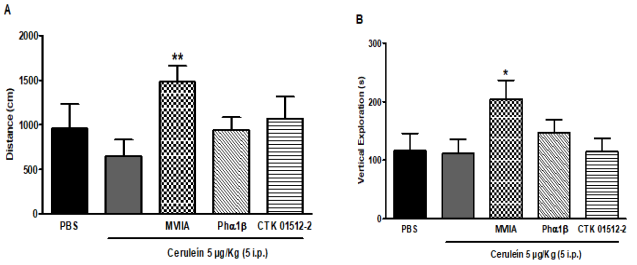

Figure 6: Evaluation of exploratory activity after 5 intraperitoneal injections of 5 µg/Kg cerulein 2 h after vehicle (PBS), Phα1β, CTK015012-2 and ω-conotoxin MVIIA.

Note: Treatments with ω-conotoxin MVIIA, Phα1β or CTK 01512-2 (100 pmol/site) by intrathecal route (n=8 per group). A: Total distance traveled in centimeters (cm). **p<0.01significance in relation to the cerulein group, Teste t de Student. B: Vertical exploratory activity in seconds (S) starting of 15 minutes. *p<0.05 significance in relation to the cerulein group, Teste t de Student.

DISCUSSION

The present study showed that CTK 01512-2 recombinant toxin attenuated visceral hyperalgesia and the inflammatory response associated with AP induced by cerulein in rats. AP-induced by cerulein is one of the models most common and accepted, being associated with nociception in experimental animals [21]. In our experimental design, five injections of cerulein 5 µg/kg (i.p.) with an interval of one hour between each were able to cause abdominal mechanical hyperalgesia within 30 to 120 minutes after the last injection of cerulein. It has been previously demonstrated that the dose of 5 µg/kg can induce AP, causing histological changes in the pancreas and serum amylase level [15]. The results also demonstrated that CTK 01512-2 recombinant toxin reduced, in a dose-dependent manner, the abdominal mechanical hyperalgesia associated with cerulein-induced AP, exhibiting similar effect to Phα1β and ω-conotoxin MVIIA. It is well known that voltage-sensitive calcium channels play an essential role in conducting the peripheral pain signal to CNS. They participate in the regulation of the excitability of the primary sensory neurons of DRG and spinal cord dorsal horn neurons, as well as the upward and downward modulation of sensory signals [22].

CTK 01512-2 toxin and MVIIA reduced ROS production in the spinal cord of animals with cerulein-induced AP. ROS play a critical role in the pathogenesis and development of AP. In acute inflammation, they originate primarily from NADPH oxidases and target the NF-κB. Activation of NF-κB and NF-κB-regulated expression of IL-1β, IL-6 and TNF-α are involved in the initiation and aggravation of AP [23].

The administration of cerulein was also able to increase serum amylase and lipase levels by more than 10 times, respectively, compared to PBS untreated. Serum levels of amylase and lipase are used as markers of pancreatic inflammation, but serum elevations of these enzymes do not always predict the presence of pancreatic disease. They may also be elevated in some other intra-abdominal diseases such as intestinal ischemia, biliary tract diseases [24]. The increase on the ROS, amylase and lipase levels were associated with mechanical abdominal hyperalgesia reinforcing the validation of the AP model used in this study. The results also demonstrated that CTK 01512-2 and Phα1β toxins reducing amylase and lipase levels and ROS ameliorating AP animals.

In contrast, the ω-conotoxin MVIIA was not able to reduce serum levels of these enzymes in animals with AP, suggesting an additional beneficial effect of CTK 1515-2 and Phα1β. Probably, this observed effect occurred because CTK 01512-2 and Phα1β toxins act reversibly and nonspecifically on voltage-sensitive calcium channels with different potencies (N>R>P/Q>L) [11] and as an antagonist of the TRPA1 receptor [12]. Research over the last decade has improved our understanding of pain mechanism in chronic pain of AP.

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One of the best-characterized pain receptors is the Transient Receptor Potential Vanilloid1 (TRPV1). TRPV1 can work alone or involving other TRP receptors and the TRPA1 to amplify the nociceptive signalling [25]. TRPA1 antagonists prevent the transition of acute chronic to chronic pancreatitis [26]. We have demonstrated the Phα1β is an antagonist of the TRPA1 receptor [12]. Thus the Phα1β may have a therapeutic action preventing the transition of AP to a state of chronic pancreatitis.

CONCLUSION

Together, our results demonstrated that CTK 01512-2 recombinant, a VSCCs toxin presented an active response profile, comparatively similar to Phα1β native toxin, in the control of nociception process in the AP model induced by cerulein in rats, suggesting the potential use of it as an analgesic drug for the treatment of pain.

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