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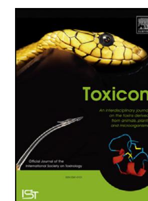

Alternagin-C (ALT-C), a disintegrin-like protein from *Rhinocerophis alternatus* snake venom promotes positive inotropism and chronotropism in fish heart

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Alternagin-C (ALT-C) is a disintegrin-like protein purified from the venom of the snake, *Rhinocerophis alternatus*. Recent studies showed that ALT-C is able to induce vascular endothelial growth factor (VEGF) expression, endothelial cell proliferation and migration, angiogenesis and to increase myoblast viability. This peptide, therefore, can play a crucial role in tissue regeneration mechanisms. The aim of this study was to evaluate the effects of a single dose of alternagin-C (0.5 mg kg^{-1}, via intra-arterial) on *in vitro* cardiac function of the freshwater fish traíra, *Hoplias malabaricus*, after 7 days. ALT-C treatment increased the cardiac performance promoting: 1) significant increases in the contraction force and in the rates of contraction and relaxation with concomitant decreases in the values of time to the peak tension and time to half- and 90% relaxation; 2) improvement in the cardiac pumping capacity and maximal electrical stimulation frequency, shifting the optimum frequency curve upward and to the right; 3) increases in myocardial VEGF levels and expression of key Ca^{2+}-cycling proteins such as SERCA (sarcoplasmic reticulum Ca^{2+}-ATPase), PLB (phospholamban), and NCX (Na^+/Ca^{2+} exchanger); 4) abolishment of the typical negative force–frequency relationship of fish myocardium. In conclusion, this study indicates that ALT-C improves cardiac function, by increasing Ca^{2+} handling efficiency leading to a positive inotropism and chronotropism. The results suggest that ALT-C may lead to better cardiac output regulation indicating its potential application in therapies for cardiac contractile dysfunction.

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1. Introduction

Disintegrins are cysteine-rich low molecular weight polypeptides isolated from snake venoms and mostly derived from proteolytically processed precursor forms having a metalloprotease domain (Fox and Serrano, 2009; Selistre-de-Araujo et al., 2010). These proteins usually contain an RGD (Arg-Gly-Asp) or KGD (Lys-Gly-Asp) sequence recognized by integrin receptors on the cell surface (Cominetti et al., 2004; Kamiguti et al., 1998). A different kind of disintegrin, called disintegrin-like, is larger than RGD disintegrins (about 30 kDa) and contains an extra C-terminal domain (Cys-rich domain) and an ECD (Glu-Cys-Asp) sequence instead of RGD (Ramos and Selistre-de-Araujo, 2006; Selistre-de-Araujo et al., 2005). Disintegrins and disintegrin-like peptides trigger integrin-mediated intracellular signal transduction events that modify gene expression and cell response and interfere with cell–cell and cell–matrix interactions in a bi-directional manner across cell membranes (Ramos et al., 2007; Teklemariam et al., 2011).

Alternagin-C (ALT-C) is an ECD-containing disintegrin-like/cysteine-rich domain released from alternagin, a metalloproteinase purified from the crude venom of the viperid snake *Rhinocerophis alternatus*, popularly known in South America as urutu. ALT-C binds to $\alpha_2\beta_1$ integrin, a major collagen receptor, competitively inhibiting cell adhesion to collagen and triggering downstream signaling molecules (Selistre-de-Araujo et al., 2005).

Previous studies demonstrated that ALT-C up-regulates vascular endothelial growth factor (VEGF) expression in fibroblasts and it induces endothelial cell proliferation (Cominetti et al., 2004; Ramos et al., 2007). ALT-C can also improve wound repair process in rat skin by increasing collagen type I deposition and fibroblast density and by stimulation of angiogenesis markedly increasing VEGF expression 7 days after injury (Sant'Ana et al., 2011; Sant'Ana et al., 2008). In addition, ALT-C increased myoblast viability in a dose-

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dependent way (Mesquita-Ferrari et al., 2009). These findings point out the significant role of the ALT-C in tissue regeneration mechanisms and its potential for therapeutic uses.

Cardiovascular disease remains the leading cause of death around the world and, consequently, there is a considerable interest in therapies and drugs able to improve the efficiency of cardiac muscle or its regeneration after severe injuries. On the other hand, integrins represent a complex family of receptors with important adhesive and signal transducer in all cells of the organism, including all cell types within the myocardium, such as fibroblasts (Manso et al., 2009; Ross, 2004). In this regard, cardiac fibroblasts can coordinate chemical, mechanical, and electrical signals within the heart through extracellular matrix/integrin interaction and secretion of cytokines and growth factors (Manso et al., 2009; Souders et al., 2009). Moreover, mechanical stress triggers signaling downstream of integrins in cardiac fibroblasts through the mediation of the focal adhesion kinase (FAK) signaling pathway which suggests that this mechanism may have a crucial role in mechanotransduction in the myocardium (Dalla Costa et al., 2010; Franchini et al., 2000). Thus, integrins can cooperatively work with growth factors and play crucial roles in myofibrillogenesis, organogenesis, and homeostasis of normal cardiac function (Ross and Borg, 2001).

An integrin-binding peptide, such as ALT-C, able to up-regulate the expression of growth factors, may be considered an interesting tool for experimental studies of cardiac function due to its potential application to the development of therapeutic strategies for cardiovascular diseases. Therefore, the purpose of the present study was to investigate the effects of ALT-C on *in vitro* assays for myocardial contractility and cardiac VEGF and Ca^{2+} handling protein expressions in traíra, *Hoplias malabaricus* (Erythrinidae), a neotropical freshwater fish species. This species is an alternative model organism to contractile function studies since its myocardium has a functional sarcoplasmic reticulum (SR) responsible for approximately 60–70% of the Ca^{2+} release to activate the myofilaments (unpublished data) similarly to humans and rabbits, while other mammals, like rats, rely on the SR over 92% of the Ca^{2+} involved in contraction (Bassani et al., 1994; Monasky and Janssen, 2009).

2. Material and methods

2.1. Animals

Sixty adult specimens of *H. malabaricus*, (Wt = 124.5 ± 9.6 g) obtained at the Santa Candida Fish Farm (Santa Cruz da Conceição, SP, Brazil) were used. In the laboratory, fish were kept in 500 l holding tanks supplied with a continuous flow of dechlorinated and aerated water (P_wO_2 > 130 mmHg) at a constant temperature (25 °C). During the acclimation period (60 days), fish were fed *ad libitum* on small live fish.

2.2. Alternagin-C

ALT-C was purified from *R. alternatus* lyophilized venom (provided by the Instituto Butantan, São Paulo, SP, Brazil) by two steps of gel filtration followed by anion exchange chromatography as previously published procedures (Souza et al., 2000).

2.3. Experimental design and treatment

Fish were randomly divided into two experimental groups with thirty animals in each one: control group, animals treated with sterile saline and ALT-C group, animals treated with 0.5 mg $kg^{-1}$of alternagin-C. The chosen dose was based on previous dose-response trial and was assumed to be an optimized dose for fish cardiac contraction.

Fish were anaesthetized in water containing 100 mg l^{-1} of benzocaine until breathing movements stopped. They were transferred to an operating table and the gills ventilated with an aerated dilute solution of benzocaine (50 mg l^{-1}). The third afferent branchial artery on the left side was cannulated (PE 10) according to the procedures previously described by Axelsson and Fritsche (1994). This cannula was filled with a solution of saline and heparin (NaCl 0.9%, 100 IU ml^{-1} of heparin) and used to deliver injections of 0.1 ml of sterile saline or ALT-C (0.5 mg kg^{-1}). To ensure complete drug delivery, after each injection the cannula was cleaned with a new solution of saline (0.2 ml) and sutured in place. After seven days, fish were killed by decapitation and the hearts were carefully excised and used for *in vitro* experiments. Other tissue samples were taken, immediately frozen into liquid nitrogen and stored at −80 °C until VEGF and western blotting analysis were carried out.

2.4. In vitro experiments

After excision, the hearts were immediately transferred to Ringer solution containing (mM): 125.0 NaCl, 2.5 KCl, 0.94 $MgSO_4$, 1.0 NaH_2PO_4, 30.0 $NaHCO_3$, 1.5 $CaCl_2$, 10 glucose and pH 7.4. Four transversal myocardial strips with a maximal thickness of 2 mm (mean length 2.90 ± 0.15 mm and mean mass 2.7 ± 0.2 mg) were excised from the ventricle and their ends were tied in two metal rings. The preparations were mounted vertically and immersed in bath containing physiological solution kept at 25 °C and continuously gassed with a mixture of 98% O_2 and 2% CO_2. One ring was attached to an isometric force transducer (Grass FT.03 Transducer, Grass Technologies, West Warwick, RI, USA) through a stainless steel wire and other was tied around platinum electrodes connected to a to Grass S88 stimulator which delivered electrical square pulses of 8 ms and a voltage 50% above that eliciting maximal twitch force.

Preparations were stretched to obtain the maximum induced force and they were allowed 40 min at 0.2 Hz (12 bpm) to stabilize before each experimental protocol (see below). Isometric contractions from the force transducers were recorded by an AcqKnowledge MP150 data-acquisition system (Biopac Systems Inc., USA). The length and wet mass of each strip were measured and isometric force (Fc) relative to cross-sectional area (mN mm^{-2}) was calculated assuming a muscle density of 1.06 mg mm^{-3} (Layland et al., 1995). Time-dependent parameters such as time-to-peak tension (TPT) and times to 50% (RT50) and 90% relaxation (RT90), and rates of tension development and relaxation (maximum derivative from the recorded force–time curve during contraction phase (+dF/dt) and during relaxation phase (-dF/dt), respectively) were also evaluated.

2.5. Protocols

The effect of stimulation frequency on the contractility of fish heart was studied by increasing pacing rate from 0.2 Hz (12 bpm) until the frequency in which at least 80% of the strips were still able to contract regularly. The cardiac pumping capacity (CPC) at each stimulation frequency was calculated as the product of Fc and heart hate as previously described (Matikainen and Vornanen, 1992).

The influence of pause on the force of contraction was measured by interrupting the normal pacing at 0.2 Hz for periods of 10, 30, 60, and 300 s. The first contraction after the pauses was compared to the last contraction in a steady-state train. The relative potentiation (ratio of post-rest contractions and steady-state contractions) phenomenon reflects the contribution of SR

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