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RESEARCH ARTICLE

Selenoprotein T as a new positive inotrope in the goldfish, *Carassius auratus*

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ABSTRACT

Selenoprotein T (SELENOT) is a thioredoxin-like protein, which mediates oxidoreductase functions via its redox active motif Cys-X-X-Sec. In mammals, SELENOT is expressed during ontogenesis and progressively decreases in adult tissues. In the heart, it is re-expressed after ischemia and induces cardioprotection against ischemia–reperfusion (IR) injury. SELENOT is present in teleost fish, including the goldfish *Carassius auratus*. This study aimed to evaluate the cardiac expression of SELENOT, and the effects of exogenous PSELT (a 43–52 SELENOT-derived peptide) on the heart function of *C. auratus*, a hypoxia tolerance fish model. We found that SELENOT was expressed in cardiac extracts of juvenile and adult fish, located in the sarcoplasmic reticulum (SR) together with calsequestrin-2. Expression increased under acute hypoxia. On *ex vivo* isolated and perfused goldfish heart preparations, under normoxia, PSELT dose dependently increased stroke volume (V_S), cardiac output ($\dot{Q}$) and stroke work (SW), involving cAMP, PKA, L-type calcium channels, SERCA2a pumps and pAkt. Under hypoxia, PSELT did not affect myocardial contractility. Only at higher concentrations (10^{-8} to 10^{-7} mol l^{-1}) was an increase of V_S and $\dot{Q}$ observed. It also reduced the cardiac expression of 3-NT, a tissue marker of nitrosative stress, which increases under low oxygen availability. These data are the first to propose SELENOT 43–52 (PSELT) as a cardiac modulator in fish, with a potential protective role under hypoxia.

KEY WORDS: SELENOT expression, Heart, Normoxia, Hypoxia, Intracellular calcium, Redox balance

INTRODUCTION

Selenoproteins are a widely distributed family of proteins characterized by the presence of selenium (Se), covalently bound within the amino acid selenocysteine (Sec; U) (Hatfield and Gladyshev, 2002). In eukaryotes, Sec is incorporated into nascent polypeptides at the UGA codon. This occurs when a specific sequence, designated as the Sec insertion sequence (SECIS), is present in the 3′-untranslated region (UTR) (Kryukov et al., 2003). Moreover, a special tRNA is needed for Sec incorporation (Lee et al., 1989; Labunskyy et al., 2014). The screening of selenoproteomes in mammalian and non-mammalian vertebrates detected 45 selenoproteins. Bony fish possess the largest selenoproteome, which includes 41 proteins (38 selenoproteins are present in zebrafish), together with several duplicated proteins (Mariotti et al., 2012).

Some selenoproteins are involved in intracellular redox homeostasis. This is the case for antioxidant proteins such as glutathione peroxidase (GPXs), thioredoxin reductase (TXNRDs) and iodothyronine deiodinases (DIOs). Other selenoproteins, which contain a conserved CXXU motif corresponding to the CXXC motif of thioredoxins, are emerging as oxido-reductases (Dikiy et al., 2007). They include selenoproteins H, M, O, V, W and T (SELENOH, SELENOM, SELENOO, SELENOV, SELENOW and SELENOT) (Brigelius-Flohé and Flohé, 2017, and references therein). SELENOT is one of the first selenoproteins identified by bioinformatic tools (Kryukov et al., 1999). Within the cell, it mainly localizes in the endoplasmic reticulum (ER) (Dikiy et al., 2007; Grumolato et al., 2008). It is highly expressed during embryogenesis and early development, but its tissue levels decline during adulthood, with the exception of the pancreas, pituitary, testis and thyroid (Tanguy et al., 2011). These observations demonstrate the instrumental role of SELENOT in endocrine and metabolic regulation (Prevost et al., 2013; Hamieh et al., 2017; Anouar et al., 2018).

SELENOT expression is modulated by several stimuli. It is up-regulated by the neurotrophic factor pituitary adenylate cyclase-activating polypeptide (PACAP) during neuroendocrine differentiation (Grumolato et al., 2003). Also, the activation of regenerative mechanisms (Tanguy et al., 2011) and post-ischemic events (Ikematsu et al., 2007) promote SELENOT expression, thus supporting a role in tissue protection against stress. Recently, it was demonstrated in the *ex vivo* rat heart that SELENOT expression increases after ischemia and that a synthetic SELENOT-derived peptide (SELENOT 43–52 or PSELT), containing the active CVSU redox motif, elicits post-conditioning myocardial protection by reducing ischemia–reperfusion (IR) injury (Rocca et al., 2018a). This involves the protective RISK (reperfusion injury salvage kinase) cascade and a reduction of apoptosis, and oxidative and nitrosative stress (Rocca et al., 2018a).

In fish, limited studies have analyzed the role of selenoproteins (Deniziak et al., 2007; Jurynec et al., 2008; Mariotti et al., 2012; Pacitti et al., 2014). Following the preliminary characterization in zebrafish of three SELENOT paralogs (Kryukov and Gladyshev, 2000; Thisse et al., 2003), three SELENOT paralogs (gfSelT1a, gfSelT1b and gfSelT2) have also been identified in several tissues of the adult goldfish, *Carassius auratus*, including the heart (Chen et al., 2017). Although a potential protective role of these proteins has been suggested (Chen et al., 2017), no information is available on the physiological significance of SELENOT in teleosts,

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particularly in relation to the modulation of cardiac performance. To fill this gap in our knowledge, we used the *C. auratus* heart to (i) evaluate the basal cardiac expression of SELENOT, (ii) ascertain whether exogenous PSELT, encompassing the active redox CXXU motif, affects the performance of the *ex vivo* isolated and perfused working heart, and (iii) identify the intracellular mechanism activated by PSELT. Moreover, as the goldfish represents a gold standard of hypoxia/anoxia resistance, able to survive at low oxygen levels thanks to a modulation of antioxidant and redox enzyme activity (Lushchak et al., 2001; Gattuso et al., 2018), we investigated whether exposure to acute hypoxia influences the cardiac expression of SELENOT and the response of the goldfish heart to PSELT.

MATERIALS AND METHODS

Animals

Specimens of the following species (both sexes) were provided by local hatcheries: goldfish, *Carassius auratus* (Linnaeus 1758) (juvenile: body length 3–5 cm, mass 3.6±0.31 g mean±s.e.m.; adult: length 12–16 cm, body mass 53.4±2.6 g); carp, *Cyprinus carpio* Linnaeus 1758 (adult: body mass 290±15 g); eel, *Anguilla anguilla* (Linnaeus 1758) (adult: body mass 95±6 g); zebrafish, *Danio rerio* (F. Hamilton 1822) (adult: body mass 0.61±0.04 g); and brown trout, *Salmo trutta* Linnaeus 1758 (adult: body mass 210 ±12 g). Goldfish used for hemodynamic studies were maintained at 18–21°C in filtered and aerated water on a 12 h:12 h light:dark cycle, and were daily fed with commercial food. Goldfish, carp, eel, zebrafish and trout used for molecular and immunofluorescence studies were killed on the day of arrival. Animals were anesthetized with MS222 (tricaine methanesulfonate; 0.2 g l^{-1}: Sigma-Aldrich) and the heart was quickly dissected out for use in the specific protocol. Animal care and experimental procedures were in accordance with Italian law (DL 27 January, 1992, no. 116) and with European Directive 2010/63/EU.

Western blot and densitometric analysis

To evaluate basal SELENOT expression, the non-perfused heart from juvenile and adult goldfish, and adult carp, eel, zebrafish and trout was removed from the pericardial cavity, and immediately frozen with liquid nitrogen. To evaluate whether heart perfusion affects SELENOT expression, the goldfish heart was removed from the perfusion apparatus after 90 min of perfusion.

Both non-perfused and perfused hearts were stored at −80°C before western blot analysis. All hearts were homogenized in ice-cold RIPA buffer (Sigma-Aldrich) containing a mixture of protease inhibitors (1 mmol l^{-1} aprotinin, 20 mmol l^{-1} phenylmethylsulfonyl fluoride and 200 mmol l^{-1} sodium orthovanadate). Homogenates were centrifuged at 10,000 ***g*** for 10 min at 4°C to remove tissue debris. Bradford reagent was used to determine protein concentration according to the manufacturer (Sigma-Aldrich); 80 µg protein sample for each homogenate was separated by SDS-PAGE on 12% (w/v) polyacrylamide gels and electroblotted onto a nitrocellulose membrane (GE Healthcare). For immunodetection, the blot was blocked with 5% non-fat dried milk for 1 h and incubated overnight at 4°C with rabbit polyclonal antibody against SELENOT (cat. no. AP53842PU-N, Acris antibodies; Hamieh et al., 2017) diluted 1:1000 in TBS-T containing 1% non-fat dried milk. Protein loading for SELENOT detection was verified by using glyceraldehyde 3-phosphate dehydrogenase (GAPDH; GAPDH polyclonal antibody cat. no. Sc-47724, Santa Cruz Biotechnology: diluted 1:20,000 in TBS-T containing 1% non-fat dried milk) in carp, eel, trout and zebrafish, and β-actin (β-actin polyclonal antibody cat. no. Sc-69879, Santa Cruz Biotechnology: diluted 1:1000 in TBS-T containing 1% non-fat dried milk) in juvenile and adult goldfish as controls. To evaluate the involvement of Akt kinase, polyclonal rabbit anti-Akt antibody (cat. no. Sc-8312, Santa Cruz Biotechnology: diluted 1:500 in TBS-T containing 1% non-fat dried milk) or polyclonal rabbit anti-pAkt1/2/3 antibody (Ser473; cat. no. Sc-7985-R, Santa Cruz Biotechnology: diluted 1:500 in TBS-T containing 1% non-fat dried milk) was used.

Peroxidase-linked secondary antibodies (anti-rabbit and anti-mouse; Santa Cruz Biotechnology) were diluted 1:2000 in TBS-T containing 5% non-fat dried milk and incubated for 1 h at RT. Immunodetection was performed using an enhanced chemiluminescence kit (ECL PLUS, GE Healthcare). Autoradiographs were scanned to obtain densitometric (arbitrary) units. Experiments were performed in triplicate and the results are expressed as means±s.e.m. of absolute values.

Immunofluorescence

For immunofluorescence, the goldfish cardiac preparations perfused under either normoxia or hypoxia, in the presence and absence of PSELT, were fixed with 4% paraformaldehyde in PBS at 4°C for 24 h and then transferred to PBS azide. Tissues were cut into 50 or 10 µm slices with a vibratome. Sections were incubated with 1% donkey serum diluted in 1% bovine serum albumin and 0.3% Triton X-100 in PBS for 2 h at room temperature, and then exposed overnight at 4°C to primary antibodies against SELENOT (Grumolato et al., 2008) diluted 1:200, anti-nitrotyrosine (3-NT), used as a marker of nitrosative stress (Merck Millipore), diluted 1:200 and calsequestrin-2, used as a marker of cardiac sarcoplasmic reticulum (SR) staining (Santa Cruz Biotechnology), diluted 1:200. Immunostaining was visualized using Alexa Fluor 488 or 594-conjugated secondary antibodies diluted 1:200 (Invitrogen). Counterstaining with 1 µg ml^{-1} 4,6-diamino-2-phenylindole (DAPI, Sigma-Aldrich) in PBS for 1 min was performed prior to mounting the slides with PBS/glycerol 50/50. Samples were analyzed with a Leica SP2 confocal laser scanning microscope (DMRAX- UV) equipped with the Acousto-Optical Beam Splitter system (Leica Microsystems). Microscopic observations were made on the cell imaging platform PRIMACEN (https://primacen.crihan.fr/).

Isolated and *in vitro* perfused goldfish hearts

The goldfish heart was removed without the parietal pericardium, cannulated and connected to a perfusion apparatus as previously described (Garofalo et al., 2012; Imbrogno et al., 2014). The heart was perfused with saline [Ringer's solution containing (in mmol l^{-1}): NaCl 124.9, KCl 2.49, $MgSO_4$ 0.94, NaH_2PO_4 1.0, glucose 5.0, $NaHCO_3$ 15.0 and $CaCl_2$ 1.2] added via the input reservoir and pumped against an afterload pressure conferred by the height of the output reservoir. For normoxic experiments, saline was equilibrated with a mixture of 99.5% O_2 and 0.5% CO_2 (Imbrogno et al., 2001); for hypoxic experiments, it was equilibrated with a mixture of 10% O_2, 0.5% CO_2 and 89.5% N_2 (Imbrogno et al., 2014). pH was adjusted to 7.7–7.9. Experiments were carried out at room temperature (18–20°C). Pressure was measured with two MP-20D pressure transducers (Micron Instruments, Simi Valley, CA, USA) connected to a PowerLab data acquisition system and analyzed using Chart software (ADInstruments, Basile, Italy); pressure was corrected for cannula resistance. Cardiac output ($\dot{Q}$) was collected over 1 min and weighed; values were corrected for fluid density and expressed as volume measurements. Heart rate (f_H) was obtained from the

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periodicity of pressure traces. Stroke volume (V_S=$\dot{Q}/f_H$) was used as a measure of ventricular performance. Ventricular stroke work [SW, mJ g^{-1}; (afterload−preload) V_S/ventricle mass] served as an index of systolic functionality.

Experimental protocols

Basal conditions

The isolated and perfused goldfish heart was allowed to maintain a spontaneous rhythm for up to 15–20 min. In all experiments, control conditions were a mean output pressure of about 1.5 kPa, with a $\dot{Q}$ set to 10–12 ml min^{-1} kg^{-1} body mass by appropriately adjusting the filling pressure (Imbrogno et al., 2014; Garofalo et al., 2012). The heart generated its own rhythm. Cardiac variables were measured simultaneously during experiments. Hearts that did not stabilize within 20 min of perfusion were discarded.

Drug application

After the 15–20 min of stabilization, the goldfish heart was perfused for 15 min with saline enriched with the SELENOT-derived peptide 43–52 (PSELT), or with the inactive peptide (inert PSELT; Sec residue replaced by Ser), at increasing concentrations (from 10^{-12} to 10^{-7} mol l^{-1}) to generate cumulative concentration–response curves. Cardiac parameters were measured after 10 min of perfusion with each drug concentration. PSELT cumulative concentration–response curves were generated under both normoxia and hypoxia.

The mechanism of action triggered by PSELT was investigated by exposing cardiac preparations to PSELT (10^{-10} mol l^{-1}) before and after treatment with MDL123330A (10^{-8} mol l^{-1}), an inhibitor of adenylate cyclase (AC); KT5720 (10^{-7} mol l^{-1}), an inhibitor of cAMP-dependent kinase (PKA); diltiazem (DLTZ: 10^{-8} mol l^{-1}), an inhibitor of L-type calcium channels; thapsigargin (THAP: 10^{-7} mol l^{-1}), an inhibitor of SERCA2a; 5-hydroxydecanoate (5-HD, 10^{-7} mol l^{-1}), an inhibitor of mitochondrial ATP-dependent potassium (K_{ATP}) channels; glibenclamide (Glib: 10^{-7} mol l^{-1}), an inhibitor of sarcolemmal and mitochondrial K_{ATP} channels.

Inhibitor concentration was selected on the basis of preliminary dose–response curves as the highest dose that did not significantly affect the goldfish cardiac performance, and also on data from the literature (see Imbrogno et al., 2004; Garofalo et al., 2009; Mazza et al., 2015; Rocca et al., 2018b). Each experiment was completed within 2 h (Imbrogno et al., 2014; Garofalo et al., 2012).

Drugs and chemicals

PSELT corresponding to the sequence FQICVSUGYR in its reduced form, and the inert PSELT without Sec were chemically synthesized by the solid phase method on a Fmoc resin as previously described (Chatenet et al., 2006) using an Applied Biosystems model 433A peptide synthesizer (AB Sciex, Courtaboeuf, France). MDL123330A, 5-HD and Glib were purchased from Sigma-Aldrich; KT5720, DLTZ and THAP were purchased from Calbiochem (VWR International, Milan, Italy). KT5720, MDL123330A and THAP were dissolved in DMSO (maximum final concentration less than 0.1%); at this concentration, DMSO alone was found to have no effect on cardiac performance (data not shown). Other drugs were dissolved in double-distilled water. All dilutions were made in Ringer's solution immediately before use.

Statistics

For data expressed as means±s.e.m. of percentage changes obtained from individual experiments, statistical analysis was determined using repeated measures ANOVA followed by Bonferroni's or Dunnett's *post hoc* test. Differences were considered statistically significant at $P<0.05$.

For densitometric analyses, values are expressed as means±s.e.m. of absolute values from individual experiments; significance was assessed by unpaired *t*-test ($P<0.05$).

GraphPad Prism software, version 4.02 (GraphPad Software Inc., San Diego, CA, USA) was used for all statistical analysis.

RESULTS

SELENOT expression in the fish heart

Basal SELENOT expression was evaluated in homogenates from non-perfused heart of juvenile and adult goldfish. Western blot analysis revealed an immunoreactive band corresponding to the approximate molecular weight of SELENOT (20 kDa) in cardiac extracts of both juvenile and adult animals (Fig. 1A). A significantly lower expression was observed in the heart from adult fish with respect to the juvenile counterpart (Fig. 1A).

To verify whether the expression of SELENOT in the adult heart is a specific trait of the goldfish, western blotting was carried out on cardiac homogenates from carp, eel, zebrafish and trout. Densitometric analysis showed that SELENOT was expressed in the cardiac extracts of all fish species examined (Fig. 1B).

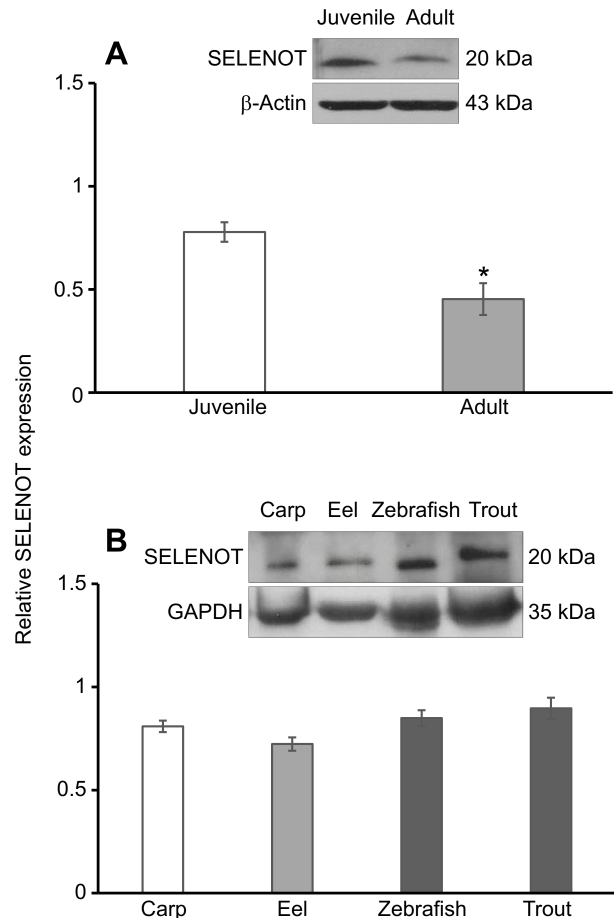

Fig. 1. Cardiac expression of selenoprotein T (SELENOT) in fish. (A) SELENOT expression (relative to that of β-actin) in non-perfused goldfish (*Carassius auratus*) hearts from juvenile (*n*=3) and adult fish (*n*=3). Significance of difference from control values (unpaired *t*-test): *$P<0.05$. Values are means±s.e.m. of three individual experiments. (B) SELENOT expression (relative to that of GAPDH) in adult carp (*Cyprinus carpio*), eel (*Anguilla anguilla*), zebrafish (*Danio rerio*) and trout (*Salmo trutta*) cardiac extracts. Insets in A and B show results of western blots.

Table 1. Baseline cardiac parameters of the isolated and perfused goldfish (*Carassius auratus*) heart under normoxia and hypoxia

	V_S (ml kg^{-1})	SW (mJ g^{-1})	f_H (beats min^{-1})	Preload (kPa)	Afterload (kPa)	$\dot{Q}$ (ml min^{-1} kg^{-1})
Normoxia	0.18±0.005	0.22±0.01	63±2.8	0.14±0.07	1.42±0.015	11.74±0.41
Hypoxia	0.162±0.07	0.27±0.02	72±1.44	0.072±0.005	1.46±0.017	11.88±0.35

V_S, stroke volume; SW, stroke work; f_H, heart rate; $\dot{Q}$, cardiac output.

Effects of PSELT on goldfish cardiac performance

Basal conditions

PSELT effects on cardiac hemodynamic performance were studied on the *ex vivo* isolated and perfused working heart of adult goldfish. Baseline variables after 15–20 min of stabilization are reported in Table 1. These conditions are comparable to those previously reported (normoxia: Garofalo et al., 2012; hypoxia: Imbrogno et al., 2014).

Effects of PSELT on cardiac performance under normoxia

To determine whether exogenous PSELT directly affects cardiac performance, the isolated and perfused goldfish heart was exposed to increasing concentrations (from 10^{-12} to 10^{-7} mol l^{-1}) of PSELT to generate concentration–response curves. PSELT dose dependently increased V_S, $\dot{Q}$ and SW at all concentrations tested (Fig. 2A–C), thus acting as a positive inotropic agent. The enhancement of myocardial contractility occurred without modifying f_H (data not shown). Preliminary experiments showed that similar responses were obtained by the repeated exposure of each heart to a single concentration of PSELT (data not shown).

To assess whether the cardiac effects elicited by PSELT were due to the Sec residue, the goldfish heart was perfused with increasing concentrations (from 10^{-12} to 10^{-7} mol l^{-1}) of inert PSELT in which the Sec residue is replaced by Ser. Within the range of concentrations used, this peptide did not affect cardiac performance (Fig. 2B–D).

Mechanism of action of PSELT

The mechanism of action of PSELT was analyzed on the isolated and perfused working goldfish heart by focusing on target proteins known

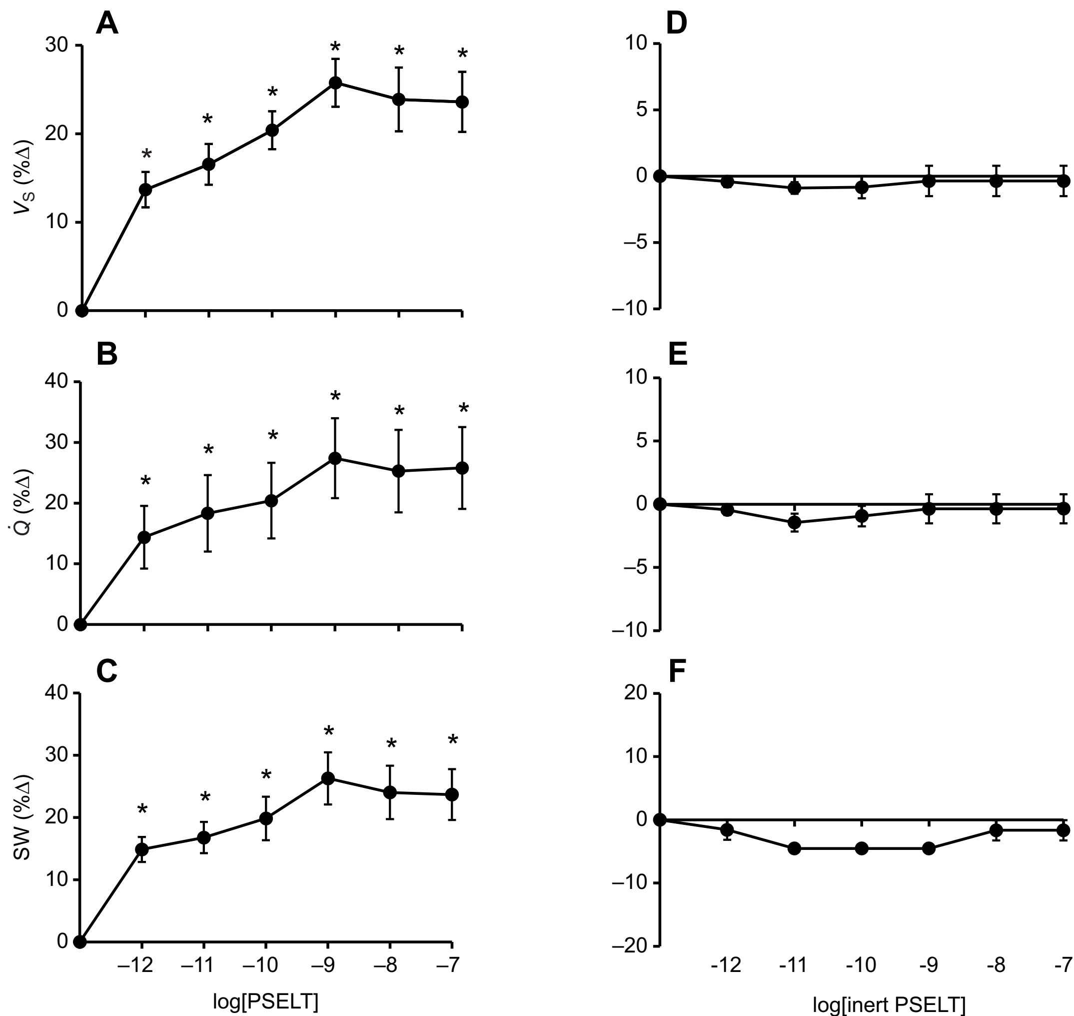

Fig. 2. Cumulative concentration–response curves for the effect of PSELT (left) and inert PSELT (right) on stroke volume, cardiac output and stroke work in the isolated working goldfish heart perfused under normoxia. (A,D) Stroke volume (V_S). (B,E) Cardiac output ($\dot{Q}$). (C,F) Stroke work (SW). Percentage change was evaluated as the mean±s.e.m. of five experiments and is plotted against log PSELT concentration (mol l^{-1}). Significance of difference from control values (repeated measures ANOVA followed by Dunnett's *post hoc* test): *P<0.05.

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to be involved in the modulation of cardiac performance in fish (e.g. Imbrogno et al., 2004; Chen et al., 2005; Garofalo et al., 2009; Mazza et al., 2015). The involvement of AC and PKA was explored by perfusing the cardiac preparations with PSELT (10^{-10} mol l^{-1}) in the presence of MDL123330A (10^{-8} mol l^{-1}), a selective AC inhibitor, and KT5720 (10^{-7} mol l^{-1}), a selective PKA blocker. Both inhibitors abolished PSELT cardiac effects (Fig. 3A).

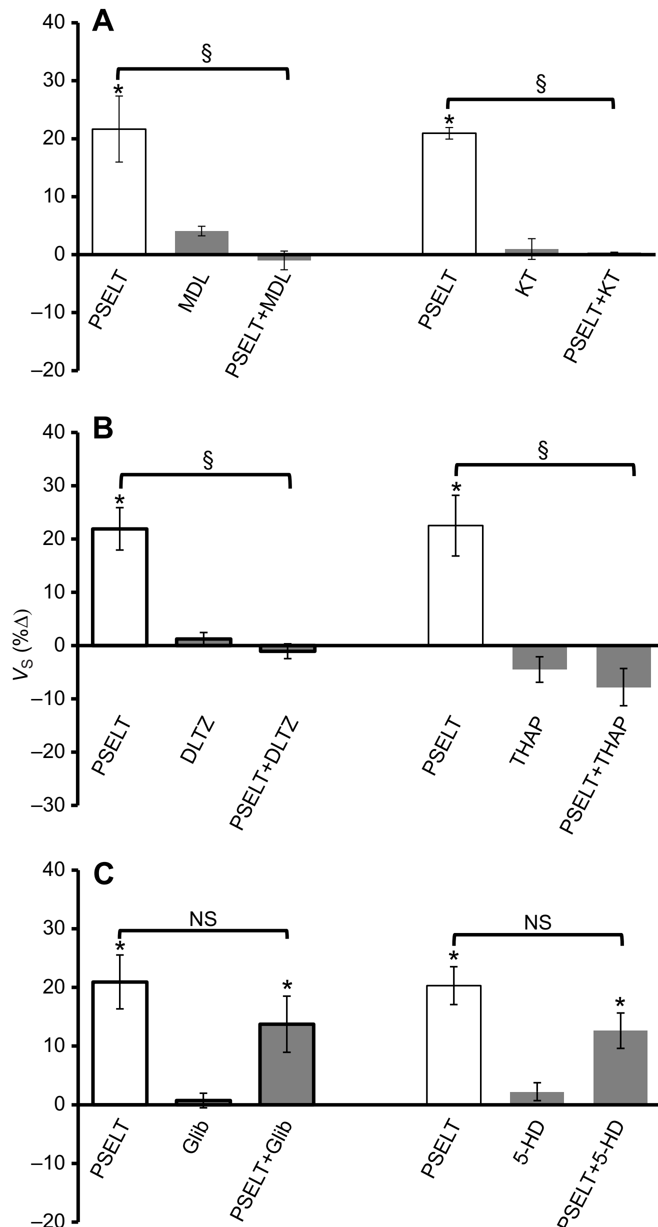

Fig. 3. Effect of PSELT (10^{-10} mol l^{-1}) before and after treatment with inhibitors on V_S of the isolated and perfused working goldfish heart. (A) Adenylate cyclase inhibitor MDL123330A (MDL, 10^{-8} mol l^{-1}) and cAMP-dependent kinase inhibitor KT5720 (KT, 10^{-7} mol l^{-1}). (B) L-Type calcium channel inhibitor diltiazem (DLTZ, 10^{-8} mol l^{-1}) and SERCA2a inhibitor thapsigargin (THAP, 10^{-7} mol l^{-1}). (C) Sarcolemmal and mitochondrial K_{ATP} channel inhibitor glibenclamide (Glib, 10^{-7} mol l^{-1}) and mitochondrial K_{ATP} channel inhibitor 5-hydroxydecanoate (5-HD, 10^{-7} mol l^{-1}). Percentage change was evaluated as the mean±s.e.m. of four experiments for each group. Significance of differences is indicated as: **P*<0.05 PSELT or PSELT+inhibitor versus control; §*P*<0.05 PSELT versus PSELT+inhibitor; NS, not significant (repeated measures ANOVA followed by Bonferroni's *post hoc* test).

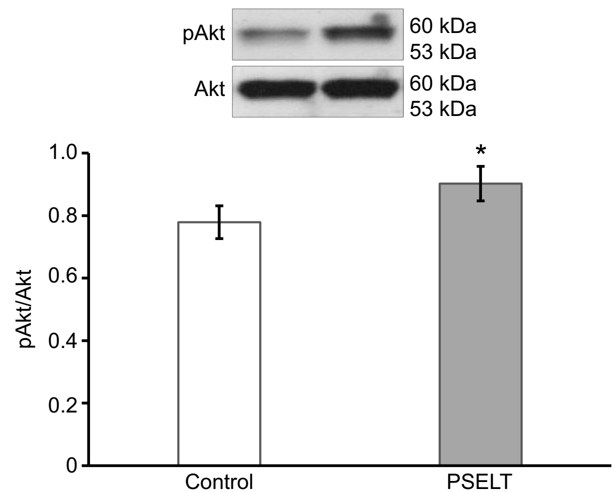

Fig. 4. p-Akt (Ser473) expression in goldfish cardiac extracts perfused with saline (control) or PSELT (10^{-10} mol l^{-1}). Values (phosphorylated versus non-phosphorylated Akt) are means±s.e.m. of three experiments for each condition. Significance of difference (unpaired *t*-test): **P*<0.05. Inset shows results of western blot.

To analyze the involvement of Ca^{2+} in the effects induced by PSELT, goldfish cardiac performance was studied before and after treatment with the L-type calcium channel antagonist DLTZ (10^{-8} mol l^{-1}) or the SERCA2a pump inhibitor THAP (10^{-7} mol l^{-1}). Both treatments abolished the stimulation of myocardial contractility induced by PSELT in the goldfish heart (Fig. 3B), implicating a Ca^{2+}-dependent mechanism.

The involvement of K_{ATP} channels in the positive effect of PSELT on goldfish cardiac contractility was evaluated by treating the cardiac preparations with either Glib (10^{-7} mol l^{-1}), a non-selective antagonist of K_{ATP} channels, or 5-HD (10^{-7} mol l^{-1}), a selective antagonist of mitochondrial K_{ATP} channels. Neither inhibitor modified the cardiac effects induced by PSELT (Fig. 3C).

Western blot analysis was used to evaluate whether Akt is involved in PSELT-induced cardiostimulation. Densitometric quantification of the blots revealed that, with respect to the basal conditions (untreated hearts), the exposure to PSELT was accompanied by increased expression of the phosphorylated form of the enzyme (pAkt) (Fig. 4).

Immunofluorescence studies by confocal microscopy revealed that, in comparison with normoxic conditions, SELENOT expression under hypoxia is stimulated in cardiac SR, as revealed by the co-labeling with calsequestrin-2 (Fig. 5). In addition, hypoxia provoked an increase in 3-NT, a marker of nitrosative stress, which was reversed by administration of PSELT (Fig. 5).

PSELT effects on cardiac performance under acute hypoxia

Under hypoxia, PSELT did not affect myocardial contractility. Only at higher concentrations (10^{-8} to 10^{-7} mol l^{-1}) was an increase of V_S and $\dot{Q}$ observed (Fig. 6).

DISCUSSION

This work is the first to show that SELENOT is expressed in the heart of both juvenile and adult goldfish, as well as in the heart of other adult teleost fish, i.e. carp, eel, zebrafish and trout. In the goldfish, SELENOT was localized in the SR and its expression was enhanced in the presence of acute hypoxia. Physio-pharmacological analyses revealed that exposure of the isolated and perfused working goldfish heart to PSELT, under normoxia, affects cardiac

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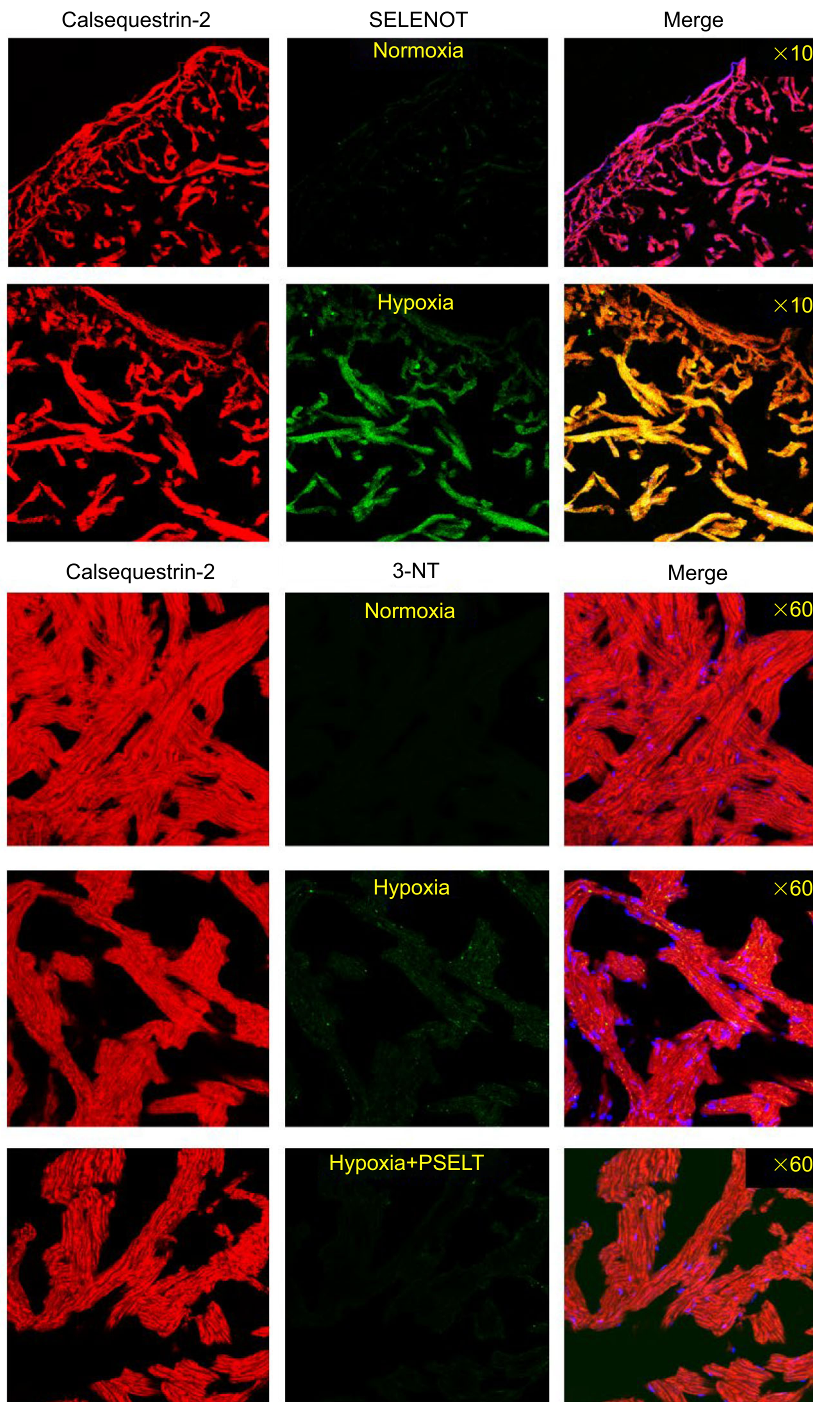

Fig. 5. SELENOT expression in the goldfish heart under normoxia and hypoxia. Top: representative images of SELENOT and calsequestrin-2 immunoreactivity under normoxia and hypoxia. Bottom: representative images of 3-NT and calsequestrin-2 immunoreactivity under normoxia, hypoxia and hypoxia in the presence of PSELT (10^{-10} mol l^{-1}).

performance by significantly increasing contractility probably via a cAMP/PKA pathway, L-type calcium channels, and SERCA2a pumps. In contrast, under hypoxia, PSELT did not significantly affect myocardial contractility, but reduced hypoxia-dependent nitrosative stress. These data suggested SELENOT is an ancient cardiac modulator, with a potential protective role under hypoxia.

SELENOT expression in non-perfused fish heart

As shown by western blot analysis, SELENOT was expressed in goldfish cardiac extracts. Expression was higher in juvenile animals, but it was maintained in adults, although at lower levels. This is different from data in mammals showing that tissue SELENOT expression is downregulated during development, being absent in adult tissues, with the exception of endocrine glands (Tanguy et al., 2011) and of tissues exposed to pathological challenges. In the rat heart, recent work by Rocca et al. (2018a) showed for the first time that SELENOT is highly expressed in embryos, decreases in newborns and is absent in adults, but it is re-expressed after IR injury. As PSELT significantly reduced several markers of IR-induced oxidative stress and apoptosis, the authors suggested a protective role of the protein under ischemic conditions (Rocca et al., 2018a).

To evaluate whether SELENOT expression in the adult heart is a trait peculiar to the goldfish, protein expression was also analyzed in

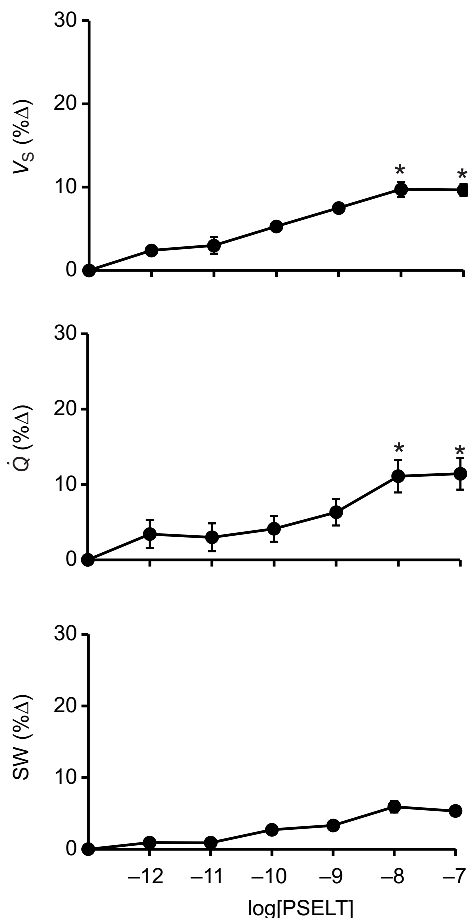

Fig. 6. Cumulative concentration–response curves for the effect of PSELT on stroke volume, cardiac output and stroke work in the isolated working goldfish heart perfused under acute hypoxia. (A) V_S; (B) $\dot{Q}$; (C) SW. Percentage change was evaluated as the mean±s.e.m. of five experiments and is plotted against log PSELT concentration (mol l^{-1}). Significance of difference from control values (repeated measures ANOVA followed by Dunnett's *post hoc* test): $^{*}P<0.05$.

cardiac extracts from other adult fish species. Western blot and densitometric analyses revealed that the protein is present in cardiac homogenates from common carp (*C. carpio*), European eel (*A. anguilla*), zebrafish (*D. rerio*) and brown trout (*S. trutta*), without significant differences among species. In fish, *SELENOT* genes are expressed in both embryos and adults. In zebrafish embryos, two orthologs of human *SELENOT* (*zSelT1a* and *zSelT1b*), plus a third gene, named *zSelT2*, with no known human ortholog (Kryukov and Gladyshev, 2000) were identified. Also in the goldfish, three *SELENOT* genes (*gfSelT1a*, *gfSelT1b* and *gfSelT2*) were cloned and their transcripts were constitutively detected in adult tissues, including the heart (Chen et al., 2017). Accordingly, it appears that, different from the developmental loss of protein expression observed in the mammalian heart (Rocca et al., 2018a), in teleost fish, the cardiac tissue retains the ability to express SELENOT during adulthood. Of note, the adult piscine heart is characterized by a high proliferative capacity relative to that of mammals and by the presence of cardiomyocytes that resemble those observed in the fetal mammalian heart (Matrone et al., 2017, and references therein). In addition, the fish heart continues to grow throughout life (Cerra et al., 2004), as well as in relation to stress stimuli (Wills et al., 2008, and references therein). This growth involves a gene program that is similar to that driving mammalian cardiac development (Matrone et al., 2017). To what extent SELENOT plays a role in the fetal-like profile of the adult fish heart deserves further attention.

PSELT as a cardioactive peptide in the goldfish

To determine whether SELENOT is able to influence the hemodynamics of the adult fish heart, isolated and *ex vivo* perfused working goldfish cardiac preparations were exposed to exogenous PSELT, a SELENOT-derived peptide (43–52), containing the active CXXU motif. Under normoxia, dose–response curves, obtained in the presence of increasing concentrations (from 10^{-12} to 10^{-7} mol l^{-1}) of the peptide, showed that PSELT stimulated myocardial contractility, acting as a positive inotropic agent. In fact, it increased V_S, $\dot{Q}$ and SW, the effect being significant starting from 10^{-12} mol l^{-1}, and with the maximum increase (V_S: 25.75±5.7%; $\dot{Q}$: 27.39±6.5%; SW: 26.28±6.2%) at 10^{-9} mol l^{-1}. These data are the first indication that in fish the active motif region of SELENOT may influence cardiac dynamics under basal conditions. In addition, in the goldfish, preparations perfused with the inert peptide lacking the Sec residue in the CXXU motif showed no changes in myocardial contractility, similar to findings in the rat heart, in which the inert peptide did not elicit any cardioprotective effect (Rocca et al., 2018a). These observations suggest that this structural feature of the protein is of great importance for its inotropic properties in the vertebrate heart.

As described by Imbrogno et al. (2014), the goldfish heart, perfused under hypoxic conditions exhibits an enhanced mechanical performance which is crucial for maintaining the functional and metabolic interactions between organs and tissues necessary for the hypoxia tolerance of the organism. In the present study, we showed by immunofluorescence that in hearts exposed to hypoxia, SELENOT expression increases with respect to hearts exposed to normoxia. This is of interest as it suggests that low oxygen levels represent a stimulus for the goldfish heart to increase SELENOT expression. It is presumable that the enhanced SELENOT production represents an intrinsic mechanism that contributes to protection of the goldfish heart against hypoxia, possibly by a modulation of the redox balance. In support of this hypothesis, immunofluorescence detection of 3-NT revealed that the increased nitrosative stress observed under acute hypoxia in the goldfish heart is reduced by exposure to PSELT. Of note, cumulative response curves (10^{-12} to 10^{-7} mol l^{-1}) revealed that, in contrast to normoxia, under hypoxia, PSELT did not affect cardiac performance, except for a slight but significant increase in myocardial contractility observed at high concentrations (10^{-8} to 10^{-7} mol l^{-1}). The reason for this reduced sensitivity is unclear. The possibility exists that the enhanced intracardiac SELENOT expression observed under oxygen deprivation lessens the efficacy of the exogenous peptide, the heart being already stimulated by endogenous SELENOT.

Evaluation of the mechanism of action showed that the positive inotropism induced by PSELT involves the cAMP/PKA pathway, L-type calcium channels and SERCA2a pumps, all known for their involvement in the modulation of cardiac performance in fish (Tibbits et al., 1991, 1992; Vornanen, 1997, 1999; Aho and Vornanen, 1998; Imbrogno et al., 2004; Garofalo et al., 2009; Mazza et al., 2015). At present, no conclusive information is available on how PSELT affects intracellular pathways. Our preliminary experiments in neuroblastoma cells (L.B. and Y.A., manuscript in preparation) showed that fluorescent PSELT is able to cross the plasma membrane. Whether this also occurs in cardiomyocytes remains to be determined.

We found that, in the normoxic goldfish heart, the increase of mechanical performance was abrogated when AC was inhibited by MDL123330A. This is in line with the role of cAMP as a mediator of the cardiostimulation elicited by nesfatin-1 in the goldfish (Mazza et al., 2015). The finding that PKA inhibition by KT5720 counteracted the cardiac stimulation induced by PSELT further supports this view.

In mammals, important downstream targets of PKA are L-type Ca^{2+} channels and SERCA2a pumps (Mattiazzi et al., 2005). For many fish species, extracellular Ca^{2+}, through sarcolemmal L-type Ca^{2+} channels and the Na^+/Ca^{2+} exchanger, plays a major role on cardiac contraction, while intracellular Ca^{2+} release from the SR contributes in a smaller and variable manner to activate cardiac contraction with species-specific and regional differences (Tibbits et al., 1991, 1992; Gesser, 1996; Vornanen, 1997, 1999; Imbrogno et al., 2004; Mazza et al., 2015). In parallel, SERCA2a pumps allow Ca^{2+} reuptake into the SR, thus controlling myocardial relaxation and internal store refilling for subsequent contraction (Aho and Vornanen, 1998; Landeira-Fernandez et al., 2004; Garofalo et al., 2009; Imbrogno et al., 2010; Mazza et al., 2015). Although the present study did not evaluate myocardial Ca^{2+} transients in the goldfish heart, it is conceivable that cardiac effects of PSELT occur by modulating intracellular Ca^{2+} cycles. In fact, application of DLTZ and THAP, L-type Ca^{2+} channel and SERCA2a pump inhibitors, respectively, abolished the increase in contractility induced by PSELT. These observations are in agreement with previous studies showing the role of SELENOT as a regulator of Ca^{2+} signaling and homeostasis in mammalian cells (Grumolato et al., 2008; Pitts and Hoffmann, 2018).

In the present study, cardiac perfusion with PSELT was also accompanied by increased phosphorylation of Akt. In mammals, Akt represents a crucial regulator of heart muscle hypertrophy and intracellular Ca^{2+} homeostasis (Chaanine and Hajjar, 2011). It protects myocardial cells, acting in concert with other effectors of the RISK cascade (Angelone et al., 2013; Perrelli et al., 2013; Filice et al., 2015). Consistent with its protective function, Akt is also involved in the cardioprotection elicited by PSELT of the rat heart exposed to IR (Rocca et al., 2018a). In non-mammalian vertebrates, the role of this kinase has received poor attention. The few available data indicate that in fish (eel: Garofalo et al., 2009; lungfish: Amelio et al., 2013) and amphibians (frog: Mazza et al., 2010, 2013, 2015), it is involved in the response of the heart to environmental, physical and chemical stimuli. These observations suggest that in the goldfish, PSELT acting in concert with Akt may contribute to the remarkable resistance to stress that characterizes the heart.

Conclusions

Using a physio-pharmacological and a bio-molecular approach, with the goldfish heart as a model, we documented for the first time that in fish SELENOT is a cardiac protein able to modulate basal cardiac contractility. The study also revealed aspects of diversity and similarity with mammals. Unlike in mammals (Rocca et al., 2018a), cardiac expression of the protein is maintained during ontogenesis, being detectable in both juvenile and adult goldfish samples. PSELT positively influenced the cardiac contractile performance of the adult goldfish and, as in mammals (Rocca et al., 2018a), this required the presence of an intact redox motif. Another similarity with mammals (Rocca et al., 2018a) is the involvement of pAkt in the intracellular mechanism of action elicited by PSELT in the goldfish heart. The recruitment of the cAMP/PKA pathway, L-type calcium channels and SERCA2a pumps for positive inotropism of PSELT suggests that the peptide acts on the goldfish myocardium via the modulation of intracellular pathways involved in the control of fish basal myocardial performance (Garofalo et al., 2009, 2015; Imbrogno et al., 2017). The present data from fish propose SELENOT as an ancient positive inotropic agent that can also be regarded as a putative new effector in the complex mechanism of hypoxia resistance of the goldfish heart, currently only partially uncovered.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: Y.A., M.C.C.; Methodology: R.M., A.G., S.I., L.B., S.L., B.Y.M., M.F., C.R., T.A.; Data curation: R.M., A.G., S.I., L.B., S.L., B.Y.M., M.F., C.R., T.A.; Writing - original draft: R.M., A.G., S.I.; Writing - review & editing: Y.A., M.C.C.; Supervision: Y.A., M.C.C.

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