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Cardiac adaptation to hypoxia: the goldfish as a natural animal model

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SOMMARIO

L'ossigeno è un gas fondamentale per la vita di molti organismi. Oltre a svolgere un ruolo principale come accettore di elettroni nella fosforilazione ossidativa, rappresenta un substrato diretto per diversi processi enzimatici *in vivo*. Sia l'uptake di ossigeno che il suo utilizzo a livello cellulare sono processi altamente controllati. Uno scarso apporto di ossigeno ai tessuti, fenomeno noto come ipossia, può portare a necrosi e/o apoptosi di diversi organi. Nell'uomo, l'ipossia può manifestarsi sia come risposta fisiologica a cambiamenti esterni (ridotta disponibilità di ossigeno in alta quota), sia come condizione patologica (anemia, insufficienza cardiaca, anomalie vascolari, malattie polmonari croniche, ecc.). Lo studio dei processi fisiologici correlati all'ipossia ha recentemente superato il confine della fisiologia umana, emergendo come argomento centrale nell'ambito della fisiologia comparata, integrativa ed ambientale. Diverse specie animali (inclusi pesci, rane, tartarughe, serpenti), sia per la loro natura, sia in risposta a cambiamenti dell'ambiente in cui vivono, si sono adattate a tollerare condizioni di ridotta (ipossia) o addirittura assente (anossia) disponibilità di ossigeno. In particolare, i pesci sono continuamente esposti a cambiamenti dei livelli di ossigeno. L'ambiente acquatico mostra infatti, rispetto all'ambiente terrestre, una variazione spaziale e temporale più ampia nel contenuto di ossigeno. Ciò è in gran parte dovuto alle proprietà intrinseche dell'acqua e alle rapide fluttuazioni in produzione e consumo di ossigeno. Tra i pesci teleostei, alcune specie di Ciprinidi del genere Carassius, come la carpa cruciana *Carassius Carassius* e il goldfish *Carassius auratus*, si classificano tra gli esempi più estremi di tolleranza all'ipossia, essendo capaci di sopravvivere a condizioni ipossiche per giorni o mesi. Contrariamente ad altri vertebrati tolleranti l'ipossia (come ad esempio la tartaruga), essi mostrano la capacità di rimanere attivi in condizioni di scarsa disponibilità di ossigeno. Questa abilità sembra essere correlata all'utilizzo di ampie riserve muscolari ed

epatiche di glicogeno, alla riduzione del metabolismo e alla loro capacità di fronteggiare i danni causati dall'aumentato stress ossidativo grazie all'incrementata espressione di enzimi antiossidanti. Inoltre, queste specie sono in grado di convertire il lattato, prodotto di scarto derivante dal metabolismo anaerobico, in etanolo, un prodotto facilmente eliminabile attraverso le branchie. Questa proprietà è legata alla presenza di una particolare isoforma dell'enzima piruvato deidrogenasi in grado di convertire il piruvato derivante dal lattato, in acetaldeide a sua volta convertita in etanolo.

La capacità di queste specie di sopravvivere e restare attive anche per lungo tempo se esposte a condizioni di ipossia o anossia sembra essere principalmente correlata alla loro straordinaria abilità di mantenere (carpa) o addirittura aumentare (goldfish) la loro performance cardiaca, favorendo in questo modo l'interazione e gli scambi tra organi e tessuti.

Partendo da queste premesse e utilizzando un approccio multidisciplinare (fisiologico, molecolare e proteomico), questo progetto di tesi ha analizzato nel goldfish *(C. auratus)*, modello naturale di resistenza all'ipossia, i meccanismi fisiologici e molecolari attivati nel cuore in risposta a bassi livelli di ossigeno, responsabili della migliorata performance meccanica, tipica di questo teleosteo. In particolar modo, è stata analizzata l'espressione e la localizzazione di effettori e modulatori intracellulari correlati alla risposta ipossica, sono stati identificati nuovi pathway trasduzionali, è stato analizzato il ruolo cardioprotettivo di enzimi antiossidanti contro il danno da ipossia ed infine sono stati analizzati meccanismi di adattamento metabolico attivati nel cuore in risposta a bassi livelli di ossigeno.

Per quanto riguarda l'attivazione di effettori cellulari un ruolo è stato attribuito ai recettori adrenergici β3. Diversi studi, principalmente condotti sui mammiferi, hanno suggerito un

coinvolgimento di questo sottotipo recettoriale nei meccanismi attivati in risposta all'ipossia. In questo studio, analisi di western blotting hanno evidenziato che il recettore β3 è espresso nel cuore del teleosteo *C. auratus* e che l'espressione aumenta in maniera significativa in condizioni ipossiche. Utilizzando un preparato di cuore di goldfish isolato e perfuso *in vitro* è stato evidenziato che in condizioni normossiche, la stimolazione di questi recettori con l'agonista BRL_{37344} genera un aumento concentrazione-dipendente della contrattilità cardiaca. Questo effetto è completamente abolito dall'utilizzo dell'antagonista dei recettori β3 (SR_{59230A}) ma non da un pool di inibitori dei recettori α/β1/β2 (fentolamina, nadololo, e ICI118,551). In condizioni ipossiche, la stimolazione dei recettori β3 non modifica la performance del goldfish, tuttavia, il trattamento con SR_{59230A} ma non con fentolamina, nadololo, e ICI118,551, abolisce completamente l'aumento della contrattilità tempo-dipendente tipica del cuore ipossico di goldfish. Sia in condizioni di normossia che in condizioni di ipossia, la via trasduzionale a valle dell'attivazione dei recettori β3 prevede il coinvolgimento dell'adenilato ciclasi (AC) e dell'adenosina monofosfato ciclico (cAMP). Questi risultati contribuiscono ad aumentare le conoscenze del controllo basale adrenergico del cuore di goldfish e propongono il recettore adrenergico β3 come un componente fondamentale dei meccanismi di tolleranza all'ipossia.

Tra i mediatori intracellulari attivati in condizioni ipossiche un ruolo è stato attribuito all'Ossido Nitrico (NO). Precedenti studi hanno evidenziato un coinvolgimento di questo trasmettitore gassoso nei meccanismi di regolazione della performance cardiaca nei pesci. Questo lavoro di tesi ha voluto analizzare nel goldfish il pathway trasduzionale attivato dallo NO in condizioni ipossiche. Utilizzando un preparato di cuore isolato e perfuso *ex vivo*, è stato osservato che l'aumento tempo dipendente della contrattilità, caratteristico del

cuore ipossico di goldfih, è abolito dall'antagonista dell'Ossido Nitrico Sintasi (NOS), N-mono-metil-L-arginina (L-NMMA), dallo scavenger dello NO, 2-fenil-4,4,5,5--tetrametilimidazoline-1-ossil-3-osside **(**PTIO), dall'inibitore della PI3-chinasi (PI3-K), Wortmannina, e dall'inibitore delle pompe SERCA2a, Tapsigargina. L'applicazione del test ELISA su cuori di goldfish perfusi in normossia e in ipossia non ha rivelato cambiamenti nei livelli di cGMP tra le due condizioni, mentre le analisi di western blotting hanno evidenziato una maggiore espressione della forma fosforilata della proteina chinasi B (pAkt) e della subunità catalitica della NADPH ossidasi, Nox2 (gp91phox). Inoltre, il saggio di Biotin Switch ha evidenziato una riduzione significativa nei livelli di proteine S-nitrosilate nei cuori di goldfish perfusi in condizioni ipossiche. I risultati ottenuti suggeriscono un'attivazione del pathway PI3K/Akt/NOS/NO e delle pompe SERCA2a nei meccanismi che contribuiscono alla migliorata performance ipossica del goldfish. L'evidente denitrosilazione proteica, associata a possibili processi di nitrazione, potrebbero rappresentare meccanismi di regolazione NO-dipendente che, attivati contemporaneamente, proteggono il cuore di goldfish dalla ridotta presenza di ossigeno.

La presente Tesi di Dottorato ha anche analizzato il ruolo cardioprotettivo di enzimi antiossidanti nei meccanismi di tolleranza all'ipossia attivati nel cuore di goldfish. È noto che, in condizioni ipossiche, vari enzimi antiossidanti svolgono un'azione protettiva riducendo il danno da stress ossidativo correlato all'ipossia. Un ruolo importante nel mantenimento dell'equilibrio redox cellulare, nonché nella cardioprotezione, è stato recentemente attribuito alla Selenoproteina T (SELENOT), una proteina con attività ossidoreduttasica. Il nostro studio ha dimostrato che il cuore di goldfish esprime questa proteina e che la sua espressione aumenta in maniera significativa in condizioni ipossiche. Analisi di immunofluorescenza hanno evidenziato che in condizioni di ridotto apporto di

ossigeno questa proteina è in grado di ridurre l'espressione cardiaca della 3-nitrotirosina, marker tissutale di stress nitrosativo, suggerendo un suo ruolo cardioprotettivo. Indagini fisio-farmacologiche condotte attraverso l'allestimento di preparati di cuore isolato e perfuso, hanno dimostrato che in condizioni normossiche, un peptide derivante dalla selenoproteina T è in grado di aumentare la performance cardiaca; questo effetto è associato ad un pathway trasduzionale che coinvolge mediatori intracellulari noti nei mammiferi per il loro ruolo nella modulazione della performance cardiaca basale e nella protezione del miocardio (cAMP, AC, PKA, Akt, canali al calcio di tipo L e pompe SERCA2a). I risultati ottenuti hanno dimostrato che la Selenoproteina T è in grado di influenzare la performance cardiaca basale del goldfish, evidenziando anche un suo ruolo nei meccanismi di resistenza all'ipossia cardiaca.

Il quarto capitolo di questa tesi riporta i risultati relativi ad uno studio proteomico, realizzato in collaborazione con la Prof.ssa A. Napoli del Dipartimento di Chimica e Tecnologie Chimiche dell'Università della Calabria, volto a valutare cambiamenti del proteoma cardiaco del goldfish in risposta a bassi livelli di ossigeno. Analisi bioinformatiche e di spettrometria di massa hanno permesso di identificare 12 proteine strettamente ricollegabili al metabolismo anaerobico e quindi all'ipossia. Tra queste, cinque sono enzimi che catalizzano fasi reversibili della glicolisi/gluconeogenesi. In particolare, è stata evidenziata la presenza di una diversa isoforma dell'enzima aldolasi in estratti cardiaci di cuori esposti ad ipossia rispetto alla controparte normossica. In letteratura sono riportate tre isoforme dell'aldolasi (A, B e C); a differenza dell'aldolasi A e C, che partecipano efficientemente al processo glicolitico, l'aldolasi B si è evoluta per avere un ruolo nella gluconeogenesi. In questo studio è stato dimostrato che mentre l'isoforma C è espressa solo in condizioni normossiche, l'isoforma B risulta espressa nei

cuori ipossici, supportando un ruolo della gluconeogenesi nei meccanismi attivati in risposta all'ipossia. In parallelo, misure dei livelli di piruvato e di lattato deidrogenasi (LDH) in omogenati di goldfish esposti ad ipossia hanno evidenziato una loro riduzione nei cuori ipossici. Questi dati suggeriscono che in condizioni ipossiche, l'attivazione dell'isoforma C dell'aldolasi permette al cuore del goldfish di riciclare il primo prodotto della glicolisi anaerobica, ovvero il piruvato, al fine di ridurre al minimo l'accumulo di lattato, e consentendo, allo stesso tempo, di evitare le conseguenze negative legate ad una bassa produzione di ATP.

Nell'insieme, i risultati riportati in questa tesi hanno evidenziato alcuni meccanismi che nel cuore di goldfish contribuiscono a sostenere la migliorata performance ipossica. In particolare, è stato dimostrato il ruolo cruciale svolto dal recettore adrenergico β3 e dal sistema NOS/NO, il ruolo protettivo esercitato da proteine antiossidanti, così come l'attivazione di alcuni meccanismi di adattamento metabolico.

Sebbene molto resti ancora da chiarire, questi risultati rappresentano una basa razionale per meglio investigare i complessi network molecolari coinvolti nella risposta cardiaca all'ipossia nei pesci. In un'ottica traslazionale, essi potrebbero anche contribuire a meglio decifrare i meccanismi che nel cuore di mammifero possono migliorare la resistenza all'ipossia, come nel caso dell'ischemia miocardica.

PREFACE

Anoxia/hypoxia tolerance in non-mammalian vertebrates

Molecular oxygen (O_2) is an essential gas for life. It represents the final electron acceptor for ATP production and the substrate for several enzymatic processes; for these reasons, an inadequate O_2 supply inhibits oxidative phosphorylation and many other oxygen-requiring reactions. In particular, a reduction in ATP levels prevents ion pumping maintenance, thus causing immediate cell damage; this consequently leads to cell depolarization, which initiates necrotic and/or apoptotic processes (Lipton, 1999). An altered ATP production is related to a shift in anaerobic metabolism, which in turn is responsible for the accumulation of harmful waste products. In mammals, this energy unbalance and others deleterious related events result, in few minutes, in cells and tissues death. Compared to mammals, several lower vertebrates (among fish, amphibians and reptiles) have evolved to tolerate a variable oxygen availability. This is largely due to a variety of specializations in body structures, lifestyles, as well as to a diversity of adaptation mechanisms to variable oxygen. Aquatic animals, more than air breathers, are likely to experience variations in oxygen ambient. In water, hypoxia results from complex converging processes which involve mixing, air-water exchange, as well as fluctuations in the pattern of O_2 production and consumption (Bograd, et al., 2008; Keeling & Garcia, 2002). Many of these processes are altered by anthropogenic and climate changes, with consequent prolonged low-oxygen conditions, and severe impact on aquatic organisms (Breitburg, et al., 2018; Diaz & Rosenberg, 2008).

Examples of hypoxia tolerance among vertebrate animals are here following provided.

Fishes

Fish exhibit a very large spectrum of oxygen sensitivity, moving from species showing an extraordinary ability to tolerate hypoxia and anoxia to species that dramatically suffer oxygen deprivation. Species that rely extensively on aerobic metabolism for rapid and sustained swimming, such as salmon and tuna, are moderately to extremely sensitive to hypoxia (Bushnell, et al., 1990; Gesser, 1977), whereas, eels and hagfish can perform well at low O_2 levels (Faust, et al., 2004). The zebrafish is characterized by a growth-dependent transition from hypoxia tolerance to sensitivity (Padilla & Roth, 2001). It has been documented that in zebrafish low oxygen exposure is associated with developmental arrest, due to a cell division block in the G and S phases of the cell cycle (Padilla & Roth, 2001). Among cyprinids, the most extreme example of hypoxia tolerance is the crucian carp (*Carassius carassius*), which is able to survive for months during hypoxia at low temperature (Nilsson & Renshaw, 2004). In addition, their cousins, the common goldfish (*Carassius auratus*), shows half-lethal times of 45 h under anoxia at 5°C and 22 h at 20°C. Contrarily to other anoxia/hypoxia tolerant species, the crucian carp and the goldfish remain active during anoxia. Both Carassius members rely on great liver and muscle glycogen reserves, reduced metabolism and avoidance of lactic acidosis by converting lactate to ethanol and CO_2. These products can be excreted through the gills, helping the animals to avoid severe acid-base disturbances (Shoubridge & Hochachka, 1980; Bickler & Buck, 2007; Fagernes, et al., 2017). Moreover, under anoxia/hypoxia, these species are able to sustain locomotor activity thanks to a myocardium contractility maintenance (Stecyk, et al., 2004a; Imbrogno, et al., 2014).

Reptiles: turtle and snake

Although turtles represent the most anoxia/hypoxia tolerant group of reptiles, not all reptiles and not even all turtles are similarly anoxia/hypoxia tolerant. The painted and red-eared turtles (genera *Chrysemus* and *Trachemys*, respectively) can survive months of complete oxygen lack during winter dormancy. While crucian carp and goldfish remain moderately active, anoxic turtles become almost comatose and contrarily to cyprinids, they can buffer large concentrations of lactic acid accumulating in the blood (Ultsch & Jackson, 1982). In fact, they are able to release calcium and magnesium carbonates from the shell to form calcium lactate, and additionally to deposit lactic acid in the shell (Jackson, 2004a; Jackson, 2004b). Also, strong bradycardia and redistribution of blood flow occurs in anoxic submerged turtles (Overgaard, et al., 2007; Stecyk, et al., 2004b). In particular, blood flow is strongly reduced to digestive organs and kidney, and increased to liver and shell. This is in line with the use of the shell for buffering and the mobilization of liver glycogen as refuelling for anaerobic metabolism.

Hypoxia tolerance has been investigated in only a few species of snakes. Garter snakes (*Thamnophis sirtalis parietalis*) survive several days without oxygen at 5◦C (Hermes-Lima. & Storey, 1993; Rice, et al., 1995). These animals are quite tolerant to hypoxia and, compared with hypoxia sensitive reptiles, possess free radical antioxidant defence mechanisms (Hermes-Lima & Zenteno-Savin, 2002).

Amphibians

In amphibians, anoxia tolerance is considered at intermediate between turtles/carp and mammals. For example, ranids survive a few days of anoxia at low temperatures (Stewart, et al., 2004) and approximately 3 h of anoxia at room temperature (Knickerbocker & Lutz, 2001). Frog larvae tolerate hypoxia better than adults of the same species (Bradford, 1983; Crowder, et al., 1998); this is consistent with the general pattern of embryonic and neonatal vertebrates more hypoxia tolerant than adults. The capacity to survive anoxia for a short time makes these species useful to investigate mechanisms of failure to prolonged anoxia tolerance. The ability to depress metabolic rate to meet ATP demands has been proposed to be crucial for frogs' survival. However, amphibians show a slow loss of ATP and dissipation of ion gradients, which also occur in the more tolerant turtles and carp but over a much longer timescale.

Strategies of hypoxia/anoxia tolerance

In hypoxia/anoxia tolerant vertebrates, the key adaptation mechanisms are related to a core triad of physiological responses which basically include: metabolic suppression, tolerance of ionic and pH disturbances, and mechanisms which avoid reoxygenation-related free-radicals injury.

Metabolic suppression

A low oxygen condition is associated with a decrease in both ATP production and utilization. To align energy demand and maintain ATP levels above a minimum, under hypoxia an organism can increase supplies through the activation of anaerobic pathways or

reduce demands. However, the ATP production *via* anaerobiosis is related to the production of waste substrates. Although substantial evidence exists supporting metabolic depression as a physiological response in the hypoxia/anoxia tolerant vertebrates, the mechanisms underlying this process are not completely understood. In particular, it is not clear whether this metabolic suppression is related to the regulation of pathways of ATP supply (i.e. glycolysis and oxidative phosphorylation), ATP demand (i.e. protein synthesis, ion pumping), or both. For example, protein synthesis and ion pumping require a lot of ATP. In particular, it is documented that in hypoxic and anoxic species, such as crucian carp and turtle, the process of protein synthesis rapidly decreases, while in hypoxia sensitive vertebrate it remains constant (Xiao, 2015). Some fish may regulate their energy production during hypoxia through the modification of regulatory enzymes of glycolysis, such as phosphofructokinase and pyruvate kinase (Rahman & Storey, 1988; Storey, 1998). Of note, in turtle tissues, the consistent phosphorylation of these enzymes is responsible for their reduced activity during hibernation (Storey, 1998). Modification of functional proteins by phosphorylation (with consequent changes in protein kinase and phosphatase activities) are used to regulate energy metabolism in the freshwater turtle, *Trachemys scripta elegans* and garter snakes, *Thamnophis sirtalis parietalis*. All these regulatory mechanisms induce a reduction in ATP demand which remains the most logical strategy to entry into a hypometabolic state.

Tolerance of ionic and pH disturbances

Ion channel arrest has been proposed as the most important step in reducing ATP demand. The documentation of channel arrest has been more difficult than that of metabolic arrest,

but there are data supporting this hypothesis. In 1986, Hochachka promoted the concept that much of the tolerance to hypoxia is a consequence of low ion permeability of cell membranes (Hochachka, 1986). In addition, it was also evidenced that in anoxic turtle hepatocytes, the membrane potential is maintained, while Na^+-K^+ ATPase activity decreases by 75% (Buck & Hochachka , 1993); a 42% decrease in voltage-gated Na^+ channel density was also described in cerebellum (Perez-Pinzon, et al., 1992). Instead, in frog skeletal muscle, a 50% decrease in Na^+-K^+ ATPase activity coupled with decreased Na^+ permeability and maintenance of membrane potential has been reported (Donohoe, et al., 2000). Also in goldfish neurons a reduction in the activity of N-methyl-d-aspartate (NMDA) receptor was reported as an effective anoxia- tolerance strategy (Bickler & Buck, 2007). Under basal conditions, the activation of NMDA receptor, a high-flux ligand-gated cation channel highly permeable to Ca^{2+}, results in a high degree of cell death. In this context, the low oxygen-dependent inhibitory regulation of this receptor results crucial for an effective anoxia-tolerance strategy in the brain.

Another important aspect activated in these species during a decrease in oxygen levels is related to their capacity to tolerate acid metabolic end products, thus reducing the changes in pH occurring during prolonged anaerobiosis. The study of Jackson (2004) has been central to revealing the role of the shell in buffering protons during prolonged anoxic dormancy in painted turtle (Jackson, 2004b). During sustained anoxia, turtles develop high plasma concentrations of both lactate and calcium which react to obtain calcium lactate. This reduces the free concentration of the acid. Furthermore, lactate is absorbed into the shell and skeleton, strongly indicating that the formation of calcium lactate also occurs in these organs. As a result, the binding of calcium to lactate leads to the efflux of lactic acid from

anoxic cells and to the use of the substantial buffering ability of the shell and skeleton. Also species from the genus *Carassius* evolved the capacity to reduce acidosis by converting lactate in the less harmful compound ethanol. Both goldfish and carp, in addition to a normal pyruvate dehydrogenase complex, possess an alternative Enzyme 1 (E1 pyruvate dehydrogenase) that is activated during anoxia and functions as an acetaldehyde-producing mitochondrial pyruvate decarboxylase (PDC). The produced acetaldehyde can then be effectively converted into ethanol by a muscle-specific alcohol dehydrogenase (ADH) (Fagernes, et al., 2017). This metabolic end-product is subsequently eliminated by diffusion across the gills into water, avoiding lactate accumulation and the related excessive metabolic acidosis.

Protection against oxidative damage

Large changes in oxygen availability, as well as the recovery from anoxia/hypoxia conditions, can lead to a wide variation in the production of reactive oxygen species (ROS). ROS are continuously produced by mitochondrial respiration and other pro-oxidants and eliminated by the antioxidant systems. In a state of equilibrium between pro-oxidants and antioxidants, the levels of oxidative damage to biomolecules are considered physiological. However, when an imbalance between pro-oxidants and antioxidants occurs, ROS levels become excessive. This excess of ROS cause oxidative stress, which is defined as non-physiological macromolecular damage and disruption of redox signaling and control (Jones, 2008). Studies on free radical stress in hypoxia-tolerant vertebrates reveal that many species constitutively express high levels of antioxidant defences. An example is represented by the constitutive expression of high levels of antioxidant enzymes and glutathione observed in

anoxic turtles (Hermes-Lima & Zenteno-Savin, 2002). In addition, other vertebrates (for example same species of fish, reptiles and amphibians) can increase the expression of antioxidant enzymes in response to hypoxia, thus preventing oxidative stress consequent to reoxygenation (Hermes-Lima & Zenteno-Savin, 2002). This strategy is used by goldfish, carp, red-sided garter snakes and leopard frogs. For example, in the goldfish exposed to anoxia, the activity of Se-glutathione peroxidase and catalase increases in the brain and in the liver, respectively, while in the carp the activity of SOD increases in liver, brain, and gill after several hours of extreme hypoxia (Vig & Nemcsok, 1989).

The heart as experimental bioassay to study hypoxia/anoxia tolerance in fish

For their formidable adaptation to hypoxia, in recent years, teleost fish, such as the common carp *C. carpio*, its related species, the crucian carp *C. carassius* and the goldfish *C. auratus,* have become widely used animal models to analyze morpho-functional adaptation to environmental or laboratory hypoxia. Information on these fascinating models of hypoxia/anoxia resistance mainly derive from studies aimed to explore the mechanisms of defence of their hearts. In fact, these species are characterized by the extraordinary capacity to retain myocardial contractility during anoxia. In particular, while in the common carp, a critical decrease in heart function occurs during 24 h of severe hypoxia, the crucian carp preserves a normal cardiac activity and autonomic cardiovascular control up to 5 days of anoxia at 8◦C (Stecyk, et al., 2004a). Interestingly, the goldfish *C. auratus* exposed to acute hypoxia shows the extraordinary ability to enhance its basal cardiac performance as well as the sensitivity to heterometric (i.e. the Frank-Starling) regulation (Imbrogno, et al., 2014). This has been considered an important mechanism for maintaining functional and

metabolic interaction between organs and tissues, required for the hypoxia tolerance of the organism (Imbrogno, et al., 2014). In fact, the preserved cardiovascular function allows the goldfish to effectively perfuse with blood both gills and liver. This mobilizes huge amount of glucose from the large hepatic glycogen store to all tissues allowing, at the same time, the transport of lactate to the muscle for ethanol conversion (Nilsson, 2001). Information is increasing on the molecular adaptations of the myocardium to hypoxia in this species. Several studies reported that the enhanced basal performance in response to hypoxia exposure is accompanied by an increased myocardial nitric oxide synthase (NOS) expression, pointing to nitric oxide (NO) generation as a crucial step for adjusting the cardiac function of the goldfish during hypoxic challenges (Imbrogno, et al., 2014). It has been also reported that the hypoxia-induced increase in NO production in goldfish heart could activate sarcolemmal K_{ATP} channels, a response that may enhance tolerance of hypoxia in this species (Cameron, et al., 2003). Of note, in the hypoxic goldfish heart, the increased NOS expression is accompanied by an enhanced expression of hypoxia inducible factor-1α (HIF-1α) (Imbrogno, et al., 2014). This hypoxia-dependent cross-talk between NOS and HIF-1α represents an evolutionary conserved trait of the vertebrate response to low O_2. Nevertheless, the mechanisms behind the hypoxia-dependent increase of cardiac contractility in the goldfish, remain still unclear.

AIM

In recent years, the research group of the Laboratory of Organ and System Physiology, where I spent my PhD period, gave a significant contribute in deciphering physiological, structural and molecular aspects of the goldfish heart. Taking advantages of their experience and of the extraordinary capacity of this animal model to face hypoxic stress, the aim of my PhD research project was to analyse physiological and molecular mechanisms activated in the goldfish heart in response to hypoxia. In particular, the project aimed to provide information about the expression and localization of intracellular effectors and/or modulators of hypoxia-related pathways, to identify new signal transduction pathways activated in response to hypoxia, as well as to investigate the role of new emerging antioxidant enzymes in eliciting cytoprotection against hypoxia injury. Moreover, a possible mechanism of cardiac metabolic adaptation in response to hypoxia has been also investigated.

After the description of the Materials and Methods used, the results obtained are here organized as follows:

Chapter 1: "Cardiac influence of the β3-adrenoceptor in the goldfish (*Carassius auratus*): a protective role under hypoxia?"

Chapter 2: "The hypoxia tolerance of the goldfish (*Carassius auratus*) heart: the NOS/NO system and beyond".

Chapter 3: "Selenoprotein T as a new positive inotrope in the goldfish, *Carassius auratus".*

Chapter 4: "MS-based proteomic analysis of cardiac response to hypoxia in the goldfish (*Carassius auratus*)".

For each chapter, a brief introduction on the state of art will be furnished.

MATERIALS AND METHODS

Animals

Specimens of goldfish (*Carassius auratus* Linnaeus 1758; body length, 12–16 cm; body weight, 46.61±3.53 g; mean ± s.e.m.) of both sexes were provided by local hatcheries. They 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. All animals were anesthetized with MS222 (tricaine methanesulfonate; 0.2 g l^{-1}: Sigma-Aldrich); the heart was quickly dissected and directed to the specific protocol. Animal care and experimental procedures were in accordance with Italian law DL 4 March 2014 no.26 (ex DL 27 January, 1992, no. 116) and with European Directive 2010/63/EU.

Isolated and perfused in vitro working heart preparations

The goldfish heart was removed without the parietal pericardium, cannulated and connected to a perfusion apparatus (Garofalo, et al., 2012; Imbrogno, et al., 2014). It received 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. 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). According with Cameron and coworker (2003) and Chen and coworker (2005), oxygen concentrations, measured in the input reservoir by using an oxygen analyser (Milwaukee, SM600), were 8.4±0.2 mg O_2 l^{-1} (normoxia) and 2.5±0.3 mg O_2 l^{-1} (hypoxia) (means±s.e.m.) (Cameron, et al., 2003; Chen, et al., 2005). Hearts were

stimulated with an LE 12006 stimulator (frequency identical to that of control, non-paced hearts; pulse width fixed at 0.1 ms; voltage, 1.2±0.1 V; mean ± s.e.m.). Pressures were measured with two MP-20D pressure transducers (Micron Instruments, Simi Valley, CA, USA) connected to a PowerLab data acquisition system and analysed by using Chart software (ADInstruments, Basile, Italy). Pressures were corrected for cannula resistance. Cardiac output (CO) was collected over 1 min and weighed. Values were corrected for fluid density and expressed as volume measurements. Heart rate (HR) was obtained from the periodicity of pressure traces. SV [expressed as CO/HR] was used as a measure of ventricular performance. Ventricular stroke work [SW; mJg^{-1}; (afterload-preload) SV/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 CO set to 10–14 ml min^{-1} kg^{-1} body mass by appropriately adjusting the filling pressure (Garofalo, et al., 2012; Imbrogno, et al., 2014). Cardiac variables were measured simultaneously during experiments. Hearts that did not stabilize within 20 min of perfusion were discarded. For time-course experiments, cardiac parameters were measured every 10 min with either normoxic or hypoxic perfusion medium, for about 90 min of perfusion.

Drug application

Cumulative concentration response curves

After the 15–20 min control period, paced hearts were perfused with Ringer's solution enriched with specific drug at increasing concentrations to generate cumulative concentration–response curves under both normoxic and hypoxic conditions. Cardiac parameters were measured after 10 min perfusion with each drug concentration.

Mechanism of action

To investigate mechanisms of action, after the stabilization period, hearts were perfused according to the following protocol: (1) perfusion with a specific drug for 10–15 min; (2) washout with Ringer's solution to return to control conditions; (3) perfusion with specific antagonist/inhibitor alone for 15–20 min; (4) perfusion with the specific drug plus the specific antagonist/inhibitor for an additional 20 min. Each experiment was completed within 2 h (Garofalo, et al., 2012).

To analyse the mechanisms involved in the time-dependent enhancement of myocardial contractility observed in the goldfish heart under acute hypoxia (Imbrogno, et al., 2014), after stabilization, cardiac preparations were perfused in the presence of specific antagonist/inhibitor for about 90 min.

Based on preliminary dose-response curves, antagonist/inhibitor concentration was the highest dose that did not significantly affect the goldfish basal cardiac performance.

Drugs and Chemicals

To study β3-AR cardiac effects and the related mechanism of action we used:

- the β3-AR agonist BRL_{37344} (from 10^{-12} to 10^{-7} mol l^{-1})
- the β3-AR antagonist, SR_{59230A} (10^{-8} mol l^{-1});
- a mixture of α/β1/β2-AR antagonists, phentolamine, nadolol and ICI118,551, respectively (10^{-7} mol l^{-1});
- the adenylyl cyclase (AC) inhibitor, MDL-12,3330A (10^{-8} mol l^{-1}).

To study the mechanism of action activated by NO we used:

- NO scavenger 2-phenyl-4,4,5,5 tetramethylimidazolineoxyl-1-oxyl-3-oxide (PTIO, 10^{-6} mol l^{-1});
- the NOS enzymes inhibitor NG monomethyl-l-arginine (L-NMMA; 10^{-5} mol l^{-1});
- the PI3-kinase (PI3-K) antagonist Wortmannin (10^{-9} mol l^{-1});
- the SERCA2a pumps inhibitor Thapsigargin (10^{-7} mol l^{-1}).

To study SELENOT cardiac effects and the related mechanism of action we used:

- SELENOT-derived peptide 43–52 (PSELT) (from 10^{-12} to 10^{-7} mol l^{-1})
- inactive peptide (inert PSELT; Sec residue replaced by Ser) (from 10^{-12} to 10^{-7} mol l^{-1})
- the AC inhibitor, MDL-12,3330A (10^{-8} mol l^{-1});

- the cAMP-dependent kinase (PKA) inhibitor, KT5720 (10^{-7} mol l^{-1});
- the inhibitor of L-type calcium channels, Diltiazem (DLTZ: 10^{-8} mol l^{-1});
- the inhibitor of SERCA2a, Thapsigargin (THAP: 10^{-7} mol l^{-1});
- the inhibitor of mitochondrial ATP-dependent potassium (KATP) channels, 5 Hydroxydecanoate (5-HD, 10^{-7} mol l^{-1});
- the inhibitor of sarcolemmal and mitochondrial KATP channels Glibenclamide (Glib: 10^{-7} mol l^{-1}).

BRL_{37344}, SR_{59230A}, phentolamine, nadolol, ICI118,551, MDL- 12,3330A, L-NMMA, 5-HD and Glib were purchased from Sigma-Aldrich. THAP, Wortmannin, PTIO, KT5720 and DLTZ were from Calbiochem. 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).

BRL_{37344}, SR_{59230A}, MDL-12,3330A, THAP, Wortmannin and KT5720 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). PTIO was dissolved in HEPES buffer (0.4 mg/mL). Other drugs were dissolved in double-distilled water. All dilutions were made in Ringer's solution immediately before use.

Western Blotting and densitometric analysis

For western blotting analysis hearts were homogenized in an ice-cold homogenization buffer (250 mmol l^{-1} sucrose, 30 mmol l^{-1} Tris-HCl, 1 mmol l^{-1} EDTA, 1% SDS, pH 7.4), containing a mixture of protease and phosphatase 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. Protein concentration in the supernatant was determined using Bradford reagent (Sigma–Aldrich) according to the manufacturer. An amount of 60-80 μg of protein sample for each homogenate was separated by SDS–PAGE on 10-12% (w/v) polyacrylamide gels and electroblotted onto a nitrocellulose membrane (GE Healthcare). For immunodetection, blot was blocked in TBS-T (TBS with 0.2% Tween-20) containing 5% non-fat dry milk and incubated overnight at 4°C with specific primary antibodies diluted in TBS-T containing non-fat dry milk. Mouse monoclonal Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) (cat# Sc-47724; dilution 1:20000) and mouse monoclonal β-actin (Santa Cruz Biotechnology, sc-69879; dilution 1:1000) antibodies were used as the loading control.

Peroxidase-linked secondary antibodies were diluted in TBS-T containing 5% non-fat dry milk and incubated for 1 h at room temperature. Immunodetection was performed by using an enhanced chemiluminescence kit (ECL PLUS, GE Healthcare). Autoradiographs were scanned to obtain arbitrary densitometric units. Experiments were performed in triplicate and the results expressed as mean±s.e.m. of absolute values.

Here following, a list of primary antibodies used is provided:

- rabbit polyclonal antibody against β3-ARs (cat# Sc-50436; dilution 1:500);

- mouse polyclonal antibody against Nox2 (cat# Sc-130543; dilution 1:1000);
- rabbit polyclonal antibodies against Akt (cat# Sc-8312; dilution 1:500);
- rabbit polyclonal antibody against pAkt [(Ser473)-pAkt1/2/3; cat# Sc-7985-R; dilution 1:500)];
- rabbit polyclonal antibody against SELENOT (cat. no. AP53842PU-N, Acris antibodies; diluted 1:1000).

Immunofluorescence

For immunofluorescence, the goldfish cardiac preparations 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 (cat. no. AP53842PU-N, Acris antibodies) (Grumolato, et al., 2008) diluted 1:200, anti-nitrotyrosine (3-NT), used as a marker of nitrosative stress (cat. no. 06-284 Merck Millipore), diluted 1:200 and calsequestrin-2, used as a marker of cardiac sarcoplasmic reticulum (SR) staining (cat# sc-390999, 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/).

cAMP/cGMP determination

For cAMP/cGMP determination hearts were treated with 5% trichloroacetic acid on ice and centrifuged at 1,500× *g* for 10 min. The supernatant was extracted three times with 5 volumes of diethyl ether saturated with water, and the aqueous phase was collected and used for cAMP/cGMP measurements by using a commercial enzyme immunoassay (cAMP and cGMP ELISA Kit; Cayman Chemical).

Biotin Switch Assay for protein s-nitrosylation assessment

Hearts perfused under normoxic and hypoxic conditions were homogenized on ice in 250 mmol l^{-1} sucrose, 30 mmol l^{-1} Tris, 1 mmol l^{-1} EDTA, 1% SDS, pH 7.4, 200 mmol l^{-1} sodium orthovanadate and Protease Inhibitor Cocktail (Sigma-Aldrich, Milan, Italy). The homogenate was centrifuged at 4 ◦C for 10 min at 10,000× *g*. The supernatant was collected, and proteins quantified with Bradford reagent. The Biotin Switch assay, performed accordingly to Jaffrey and Snyder (Jaffrey & Snyder, 2001), basically includes three steps. Briefly, in the first step, free thiols were blocked by incubation with the thiol-specific methylthiolating agent methyl methanethiosulfonate (MMTS). After the blocking of free thiols, nitrosothiol bonds were selectively decomposed with ascorbate. In the last step, the newly formed thiols were reacted with N-[6-(biotinamido)hexyl]-3′-(2′-pyridyldithio)propionamide (biotin-HPDP), a sulfhydryl-specific biotinylating reagent. MMTS were completely removed by acetone precipitation before treatment with biotin-

HPDP. Labeled proteins (60 μg of total protein) were separated on 10% (w/v) polyacrylamide gels by SDS–PAGE, transferred to nitrocellulose membrane, blocked with non-fat dried milk, and incubated with streptavidin-peroxidase diluted to 1:1,000 for 1 h. For immunodetection, an enhanced chemiluminescence kit (ECL PLUS, GE Healthcare) was used.

SDS-PAGE for proteomic analysis

Hearts perfused in normoxic and hypoxic conditions were weighed, shredded, and equally divided. One half was homogenized in 0,05 M ammonium bicarbonate [pH 8.0; 1:10 (w/v)], the other one in RIPA buffer (Sigma Aldrich) [pH 8.0; 1:10 (w/v)], by using a glass potter homogenizing vessel with a Teflon pestle on ice. Homogenates were centrifuged at 1,000 × *g* for 10 min at 4 °C to remove tissue debris. After centrifugation the supernatant was collected and Bradford reagent was used to determine protein concentration according to the manufacturer (Sigma–Aldrich). Amounts of 4 μg protein of each tissue were separated on SDS/12% (w/v) polyacrylamide gel and then stained in blu comassie for mass spectrometry analysis.

Mass spectrometry

Mass spectrometry analyses were performed using a 5800 MALDI-TOF-TOF Analyzer (AB SCIEX, Darmstadt, Germany), using a neodymium: yttrium-aluminum-garnet laser (349 nm), in reflectron positive mode with a mass accuracy of 5 ppm. The adopted experimental conditions were already reported (Aiello, et al., 2014). Briefly, 4,000 laser shots were typically accumulated with a laser pulse rate of 400 Hz in the MS mode, whereas in the

MS/MS mode spectra up to 5,000 laser shots were acquired and averaged with a pulse rate of 1000 Hz. MS/MS experiments were performed at a collision energy of 1 kV; ambient air was used as collision gas with medium pressure of 10^{-6} Torr**.**

Linear MALDI

1 µL of sample solution (80 nmol/µL, normoxic and hypoxic conditions) was mixed with 2 µL of α-cyano-4-hydroxycinnamic acid (CHCA) [5 mg/mL; H_2O/CH_3CN, 40:60 (v/v) with 0.3% TFA]. A 1 µL portion of sample–matrix solution was spotted on a MALDI plate and dried at room temperature. Linear MALDI MS spectra were acquired averaging 4,000 laser shots with a mass accuracy of 500 ppm in default calibration mode that was performed using the following set of standards: aldolase (rabbit, [M + H] + avg = 39,905), BSA (bovin serum albumin [M +H]+ avg= 66,431) and IgG1 (murine myeloma [M +H] +avg= 148,500) (Napoli, et al., 2006).

Mass spectrometry data processing

Protein identification was performed with the Protein Pilot 4.0 software program (AB SCIEX) using the Paragon (AB SCIEX) protein database search algorithm. The data analysis parameters were as follows: Sample type: Identification; Cys Alkylation: None; Digestion: Trypsin; Instrument: 5800; Special factors: Phosphorylation emphasis; Species: None; ID Focus: Biological modifications –Amino acid substitution; Database: uniport taxonomy_Actiinopterigji_7898.fasta; Search Effort: Thorough ID; FDR analysis: Yes; Detected Protein Threshold [Unused ProtScore (Conf)]: 1.5 (95.0%). After acquisition, spectra were handled using Data Explorer version 4.11 (AB Sciex). The MS/MS data were

also processed to assign candidate peptides in the NCBI, SwissProt database using the MASCOT search program (http://www.matrixscience.com). The mass tolerance of the parent and fragments for MS/MS data search was set at 10 ppm and 0.20 Da, respectively. The query was made for "the ray-finned fishes (Actinopterygii)" taxonomy allowing 2 missed cleavage. A Peaklist of 50 ions of intensity higher 10% above the noise level were generically used for database search. The phosphorylation of serine and threonine was included in the variable modifications. Most of MS/MS spectra showed intense and well resolved ion signals. However, all spectra were manually checked to verify the validity of the MASCOT results (Aiello, et al., 2017; Aiello, et al., 2020)

Functional and gene ontology (GO) analysis

The list of non-redundant protein IDs was subjected to PANTHER program (http://www.pantherdb.org/) to identifying protein class and biological process. The whole *Danio rerio* genome was selected as a reference set since the goldfish genome is not completely sequenced. Functional Annotation Clustering were obtained using DAVID software (http://david.abcc.ncifcrf.gov/). For that, all protein entries were processed indicating the corresponding zebrafish reference entry (ZIFN ID, https://www.uniprot.org/) UniProtKB.

Network analysis

Network analysis was performed submitting the orthologous *Danio rerio* gene IDs to the STRING (Search Tool for the Retrieval of Interacting Genes) software (v.11) (http://stringdb.org/) (Szklarczyk, et al., 2011). This is a large database of known and

predicted protein interactions. Proteins were represented with nodes; direct (physical) interactions were represented with continuous lines, while indirect (functional) interactions by dashed lines. All the edges were supported by at least a reference from the literature or from canonical information stored in the STRING dataset. A confidence score was fixed to 0.7 (high confidence). Cluster networks were created using the MCL algorithm which is included in the STRING website and a value of 3 was selected for all the analyses.

Biochemical assays

Sample preparation

Perfused goldfish hearts were homogenized in ice-cold homogenization buffer (EDTA 0,025 M, NaCl 0,01 M, Tris-HCl 0,01 M pH 7.4, Triton 0,1%) containing a mixture of protease and phosphatase inhibitors (1 mmol l^{-1} aprotinin, 20 mmol l^{-1} phenylmethylsulfonyl fluoride, and 200 mmol l^{-1} sodium ortho vanadate) and centrifuged at 10,000 g for 10 min at 4 °C. Cytosolic fraction was collected and protein concentration determined with Bradford reagent.

Pyruvate assay

Tissue pyruvate measurements were performed as described by Hegde and coworkers (Hegde, et al., 2010). Briefly, cardiac homogenates were mixed with NADH and LDH reagent, and pyruvate concentration was enzymatically determined by monitoring spectrophotometrically the decrease in absorption (at 340 nm) due to the NADH-dependent reduction of pyruvate to lactate by LDH. The assay was done as follows: a reagent mixture consisting of 0.2 mM NADH in 0.1 M phosphate buffer, pH 7.5, and LDH (10 units) was

prepared and the initial absorption (OD1) noted; 70 µl of the cardiac extract was then added to the reaction mixture (3 ml) and OD2 was read after 5 min. The temperature was maintained at 25°C. Pyruvate concentration, represented by the difference in absorption (OD1–OD2), was calculated with reference to the extinction coefficient of NADH and expressed as µmol/mg protein.

Lactate dehydrogenase assay

LDH was determined by the method described by Lim and coworkers (Lim, et al., 1975). For assay of lactate dehydrogenase activity, the oxidation of NADH was followed by measuring the rate of decrease in absorbance at 340 nm in a 3 ml reaction mixture containing potassium phosphate (0.1 M, pH 7.5), sodium pyruvate (0.66 mM) and NADH (0.2 mM), supplemented with 2 µL of tissue supernatant. Absorbance changes at 340 nm were measured with a Jasco V-530 UV/VIS spectrophotometer. The assay was performed at 25°C. LDH activity was expressed as UI/mg protein.

Statistics

For physiological experiments, data were expressed as means±s.e.m. of percentage changes obtained from individual experiments. Statistical analysis was determined by using unpaired t-test, or one-way ANOVA, or repeated measures ANOVA followed by Bonferroni's or Dunnett's post-test. Differences were considered statistically significant at *$p<0.05$. For densitometric analyses and cAMP determination, values were expressed as means±s.e.m. of absolute values from individual experiments; statistics was assessed by unpaired t-test. Significance was concluded at *$p<0.05$. Biochemical assay experiments were

performed in triplicate; data were expressed as means±s.e.m. of absolute values from individual experiments. Statistics was assessed by unpaired t-test. Significance was concluded at *$p<0.05$. GraphPad Prism software, v.4.02 (GraphPad Software Inc., San Diego, CA, USA) was used for the statistical analysis.

CHAPTER 1.

Cardiac influence of the β3-adrenoceptor in the goldfish (Carassius auratus): a protective role under hypoxia?

1.1. INTRODUCTION

Since the studies by Otto Loewi (Loewi, 1921), a functional catecholamine-(CA)-adrenergic receptor (ARs) axis which allows the heart to face the systemic demands required by the stress response has been established. It is well known that the vertebrate heart receives a catecholaminergic control. CAs (either circulating, or locally secreted by CA-containing chromaffin cells, or released by sympathetic terminals) reach the heart activating a pool of cardiac ARs. These include both β1 and β2 receptors, responsible for most of the cardiac adrenergic responses such as positive chronotropic, inotropic and lusitropic effects (Brodde, 1991). As in the mammalian heart, also in teleost species, CAs elicit an essentially excitatory tone, mediated by β-ARs (Ask, et al., 1980; Axelsson, et al., 1987; Cameron, 1979; Gamperl, et al., 1994). Under resting and normoxic conditions, this basal adrenergic tone is generally lesser than the cholinergic one (varying from 5 to 30%, depending on species and on temperature, and complemented by circulating CAs) (Taylor, et al., 2014). However, with some exceptions amongst species (e.g. the stress tolerant species *Anguilla anguilla*), CAs increase in response to acute physiological or environmental stress such as exercise, hypoxia, acidosis and hyperkalemia (Hanson, et al., 2006; Randall & Perry, 1992; Reid, et al., 1998).

About 30 years after the discovery of both β1 and β2 receptors, cardiovascular research led to the identification of a third β-AR subtype. This receptor, originally considered "atypical", strongly questioned the almost exclusive cardiac role of β1- and β2-ARs and was named β3-AR (Emorine, et al., 1989). As β1- and β2-ARs, also β3 is a G-protein coupled receptor (GPCR) and has been detected in atrial and ventricular extracts of the mammalian heart

(Gauthier, et al., 2000; Myslivecek, et al., 2006). Its cardiac response differs not only among the species but also on the anatomical region within the myocardium (Imbrogno, et al., 2015). Interestingly, a number of studies performed in mammals support its cardioprotective properties exerted through autocrine, paracrine and systemic effects (Balligand, 2016). Recently, the β3-AR has emerged as a potential target for the treatment of ischemic disorders because of its upregulation in response to hypoxia. For example, in cardiac and vascular tissues affected by ischemic/hypoxic conditions, β3-ARs expression significantly increases (Dessy & Balligand, 2010). Studies performed by Dal Monte and co-workers have been critical in demonstrating the crucial relationship between hypoxia and β3-ARs; particularly, these studies evidenced that β3-AR expression markedly increases in mouse B16F10 melanoma cells (Dal Monte, et al., 2013a), as well as in *ex vivo* mammalian retinal explants exposed to hypoxia (Dal Monte, et al., 2013b; Dal Monte, et al., 2012; Ristori, et al., 2011).

In the last two decades, also in the teleost heart, the potential role of the cardiac β3-AR subtype in controlling heart function received a lot of attention. First evidence on the presence of β3-ARs in the teleost heart derives from studies on the trout (*Oncorhynchus mykiss*) myocardium (Nickerson, et al., 2003). The stimulation of β3-ARs has been shown to depress contractility in the eel (Imbrogno, et al., 2006), rainbow trout (Petersen, et al., 2013) and common carp (Petersen, et al., 2015), whereas a positive inotropic effect is reported in the channel catfish (Petersen, et al., 2015). Interestingly, a recent study by Motika and co-workers reported a correlation between hypoxia and β3-ARs in the fish heart (Motyka, et al., 2017). It has been documented that acclimation to moderate chronic hypoxia (~40% air

saturation for 17-23 weeks) elicits a reduced pumping capacity of the hypoxia-sensitive trout (*O. mykiss*) heart, which correlates with a loss of cardiac β3-ARs (Motyka, et al., 2017).

However, no information is available about the expression and the potential role of β3-ARs in the modulation of cardiac performance in a model of hypoxia resistant fish, such as the goldfish. On this background, we firstly analysed the basal cardiac expression of β3-ARs and then focused the attention on its possible involvement in the hypoxia-dependent improved pumping capacity which characterizes the hypoxic goldfish heart and the signal transduction pathway activated.

1.2. RESULTS

Published in: *J. Exp. Biol. (2019) 222.* ***Leo, S.;*** *Gattuso, A.; Mazza, R.; Filice,M.; Cerra, M.C.; Imbrogno, S. Cardiac influence of the beta3-adrenoceptor in the goldfish (Carassius auratus): A protective role under hypoxia?*

1.2.1. Cardiac β3-AR expression

Basal cardiac β3-ARs expression was evaluated in homogenates from non-perfused and perfused hearts under both normoxic and hypoxic conditions. Western blottling analysis revealed an immunoreactive band corresponding to the approximate molecular weight of β3-ARs (44 kDa) in the cardiac extracts from all experimental conditions. A significant increase of β3-ARs expression was observed following acute hypoxia (Figure 1).

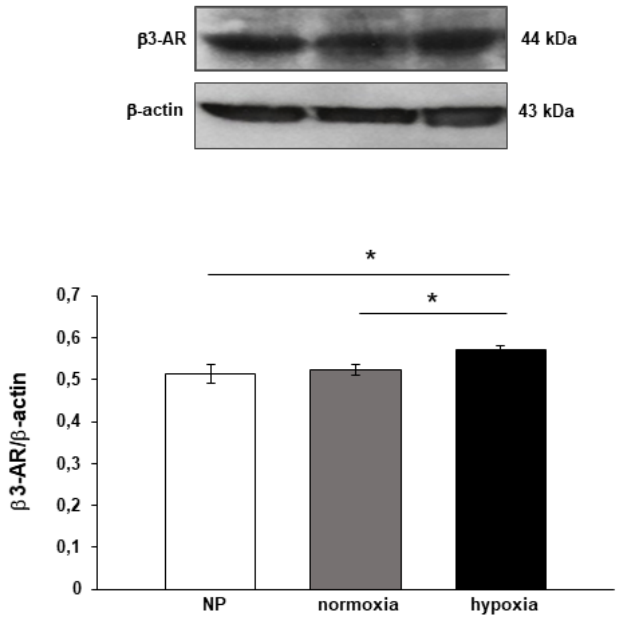

Figure 1. *β3-AR protein levels (relative to that of β-actin) in non-perfused (NP) and perfused hearts under either normoxia or hypoxia. Significance of differences from control values (unpaired t-test): *p<0.05. Values are means ± s.e.m. of three experiments for each condition*

1.2.2. Isolated heart preparation

After stabilization, the *ex vivo* isolated and perfused goldfish heart preparation values of preload, afterload, HR, CO, SV and SW (Table 1) were comparable to those previously reported [normoxia (Imbrogno & Cerra, 2017; Mazza, et al., 2015); hypoxia (Imbrogno, et al., 2014)].

Table 1. *Baseline cardiac parameters of the isolated and perfused goldfish (Carassius auratus) heart under normoxia and hypoxia; Values are means ± s.e.m. of n=19 for normoxia and n=15 for hypoxia.*

	CO (ml min^{-1} kg^{-1})	**SV** (ml kg^{-1})	***HR*** (beats min^{-1})	**SW** (mJ g^{-1})	**Preload** (kPa)	**Afterload** (kPa)
Normoxia	12.86±0.29	0.19±0.014	71.75±2.24	0.26±0.02	0.11±0.012	1.42±0.01
Hypoxia	13.31±0.68	0.18±0.014	75.11±3.75	0.29±0.02	0.06±0.005	1.44±0.005

1.2.3. Effects of β3-AR stimulation under normoxia

1.2.3.1. Dose–response curve of BRL_{37344}

To analyse the putative effects of β3-AR stimulation under basal conditions, isolated hearts perfused under normoxia were exposed to increasing concentrations (from 10^{-12} to 10^{-7} mol l^{-1}) of the β3-AR agonist BRL_{37344}. The agonist induced a significant dose-dependent increase of myocardial contractility starting from the concentration of 10^{-10} mol l^{-1} (Figure 2).

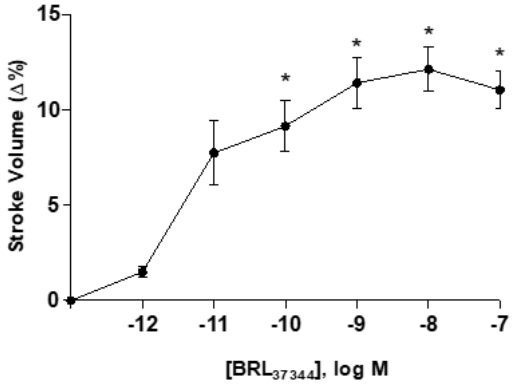

Figure 2. *Cumulative dose–response curve of BRL_{37344} on stroke volume in isolated and perfused working goldfish heart. Percentage changes were evaluated as means ± s.e.m. of 6 experiments. Significance of difference from control values (repeated measures ANOVA; Dunnett's post hoc test): *p<0.05.*

1.2.3.2. Effects of BRL_{37344} after treatment with adrenergic antagonists

To investigate if the BRL_{37344}-dependent increase of contractility specifically enrols β3-ARs, isolated and perfused goldfish hearts were treated with BRL_{37344} (10^{-9} mol l^{-1}) in the presence of a specific β3-AR antagonist (SR_{59230A}; 10^{-8} mol l^{-1}), or a mixture of α/β1/β2-AR inhibitors (phentolamine, nadolol and ICI118,551, respectively; 10^{-7} mol l^{-1}). The effect of BRL_{37344} was abolished by SR_{59230A}, whereas it was unaffected by α/β1/β2-AR antagonists (Figure 3A,B).

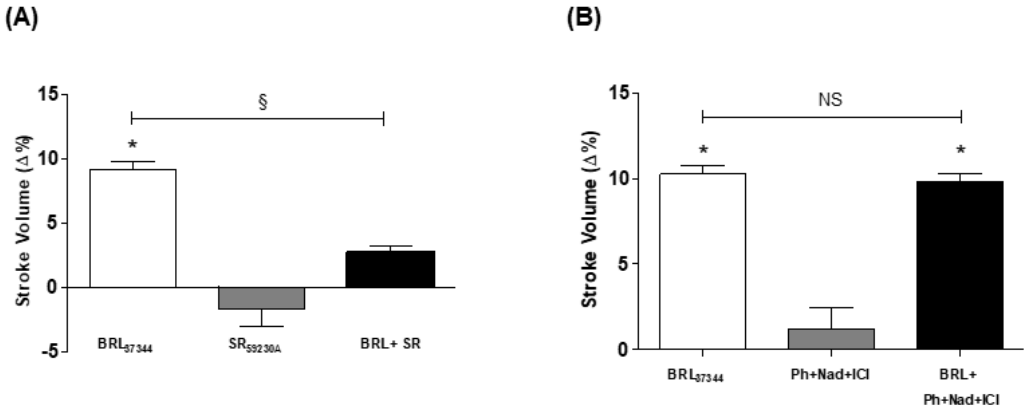

Figure 3. *BRL_{37344} (10^{-9} mol l^{-1}) effects before and after treatment with* ***(A)*** *SR_{59230A} (10^{-8} mol l^{-1}) and* ***(B)*** *a mixture of a/β1/β2 antagonists (10^{-7} mol l^{-1}). Percentage changes were evaluated as means ± s.e.m. of 4–5 experiments for each group. Repeated measures ANOVA followed by Bonferroni's post hoc test; *p<0.05 (BRL or BRL+antagonists versus control); §p<0.05 (BRL versus BRL+ SR). NS, not significant. BRL, BRL_{37344}; ICI, ICI118,551; Nad, nadolol; Ph, phentolamine; SR, SR_{59230A}.*

1.2.3.3. Involvement of the adenylyl cyclase/cAMP signal transduction pathway

To investigate the involvement of the AC-mediated pathway in the mechanism of action activated by β3-ARs, isolated and perfused goldfish hearts were pre-treated with a specific AC inhibitor (MDL-12,3330A; 10^{-8} mol l^{-1}). MDL-12,3330A treatment abolished the increase of contractility elicited by BRL_{37344}. This effect was accompanied by an enhancement of cAMP concentration observed in the goldfish hearts treated with BRL_{37344} (Figure 4A,B).

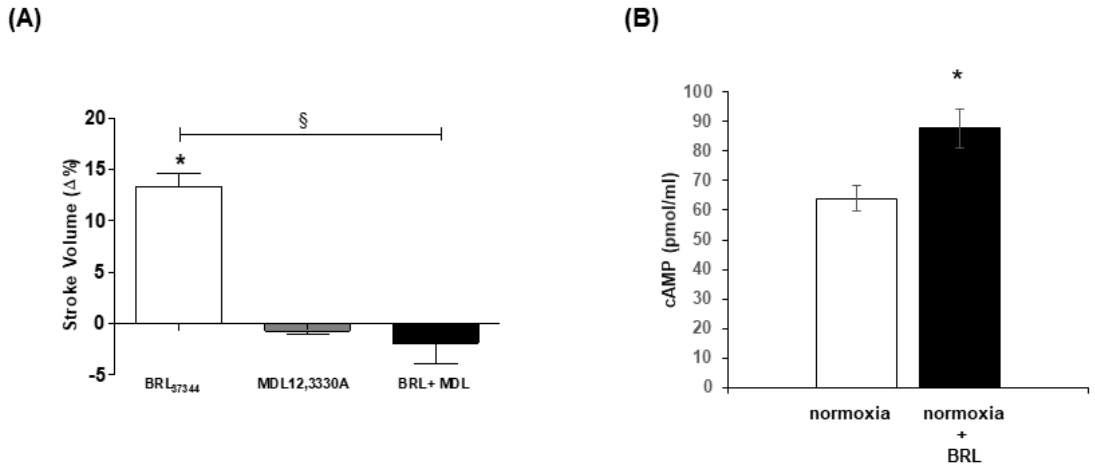

Figure 4. (A) *Effects of BRL_{37344} (10^{-9} mol l^{-1}) on stroke volume before and after treatment with MDL-12,3330A (10^{-8} mol l^{-1}). Percentage changes were evaluated as means ± s.e.m. of 4 experiments. Differences are indicated as: *p<0.05 (BRL versus control), §p<0.05 (BRL versus BRL+inhibitor); repeated measures ANOVA followed by Bonferroni's post hoc test.* ***(B)*** *cAMP levels in goldfish cardiac extracts under normoxia with and without BRL_{37344} treatment. Significance of difference from control values (unpaired t-test): *p<0.05 (n=3). BRL, BRL_{37344}; MDL, MDL-12,3330A.*

1.2.4. Role of β3-ARs in the hypoxia-induced increase of contractility

1.2.4.1. Dose-response curve of BRL_{37344}

In contrast to normoxia, under acute hypoxia, BRL_{37344} (from 10^{-12} to 10^{-7} mol l^{-1}) did not significantly affect the goldfish heart performance (data not shown).

1.2.4.2. Effects of adrenergic antagonists

As shown by Imbrogno and co-workers, exposure of the isolated and perfused goldfish heart to a hypoxic medium is accompanied by a time-dependent enhancement of the mechanical performance (Imbrogno, et al., 2014). This effect was abolished by treatment with the specific β3-AR antagonist SR_{59230A} (10^{-8} mol l^{-1}), but not by the mixture of α/β1/β2-AR inhibitors (10^{-7} mol l^{-1}) (Figure 5).

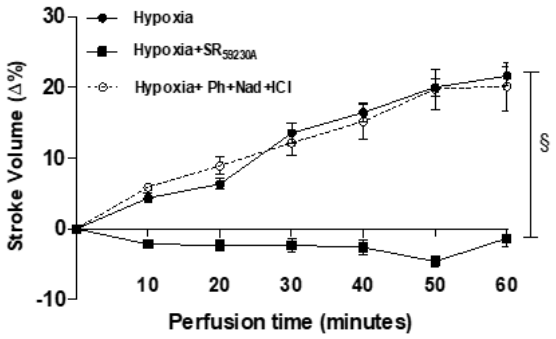

Figure 5. *Time-course curves of stroke volume before and after treatment with SR_{59230A} (10^{-8} mol l^{-1}) and with a mixture of a/β1/β2 AR antagonists (10^{-7} mol l^{-1}). Data are expressed as means ± s.e.m. of 4 experiments for each group. Differences are indicated as: §p<0.05; one-way ANOVA followed by Bonferroni's post hoc test. ICI, ICI118,551; Nad, nadolol; Ph, phentolamine; SR, SR_{59230A}.*

1.2.4.3. Involvement of the adenylate cyclase/cAMP signal transduction pathway

As in normoxia, under hypoxia, the transduction pathway activated by β3-ARs was also examined by perfusing cardiac preparations with the AC inhibitor MDL-12,3330A (10^{-8} mol l^{-1}). The treatment abolished the hypoxia-dependent increase of contractility, demonstrating its dependence on the AC activation. This was confirmed by the detection of increased concentrations of cAMP in extracts of goldfish hearts perfused with the hypoxic medium (Figure 6A,B).

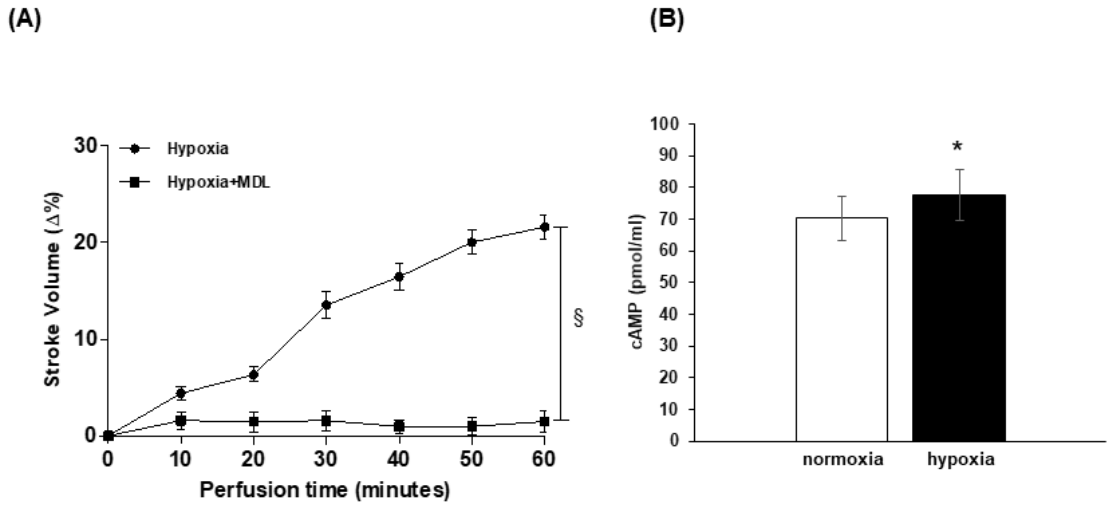

Figure 6. (A) *Time course curves of stroke volume before and after treatment with MDL-12,3330A (10^{-8} mol l^{-1}). Data are expressed as mean values ± s.e.m. of 3 experiments. Differences are indicated as: §p<0.05; two-tailed unpaired t-test.* ***(B)*** *cAMP levels in goldfish cardiac extracts under normoxia and hypoxia. Significance of difference from control values (unpaired t-test): *p<0.05 (n=3).*

1.3. DISCUSSION

The present study is the first to analyse the expression of β3-ARs in the goldfish heart and to propose a role for this adrenoceptor in the modulation of the cardiac performance under both normoxic and hypoxic conditions.

1.3.1. β3-AR expression in the normoxic and hypoxic goldfish heart

In mammals, the heart is a major β3-AR-expressing organ. The receptor localizes on both the myocardium and the coronary endothelium (Dessy, et al., 2004), and plays a role in cardiac function and remodelling (Balligand, 2016; Imbrogno, et al., 2015). Currently, very few studies document the presence of β3 ARs in the teleost heart. In 2003, Nickerson and co-workers detected two β-ARs in *O. mykiss* (adrb3a, NP_001118100; adrb3b, NP_00117924) (Nickerson, et al., 2003). These β-ARs are homologous to the mammalian β3-AR and are highly expressed in the heart. Subsequently, sequences similar to β3-ARs were identified in various teleost species, including zebrafish (adrb3a: BAH84778 and adrb3b: NP_001128606), black bullhead (adrb3b: ABH10580), stickleback (ENSGACP00000014582) and fugu (ENSTRUP 00000020 757) [for references, see (Imbrogno, et al., 2015)]. In the present study, we found that β3-ARs are expressed in the goldfish heart. A significant increased expression was observed in hearts exposed to hypoxia compared with both non-perfused hearts and hearts perfused under normoxia. These results are in line with the upregulation of β3-ARs observed in *ex vivo* mouse retinal explants exposed to low O_2 (Dal Monte, et al., 2013b). However, they conflict with observations in the hypoxia-sensitive trout heart, where the hypoxia-dependent reduced pumping capacity has been attributed to a loss of cardiac β3-ARs (Motyka, et al., 2017). It cannot be excluded that in fish the influence of hypoxia on

cardiac β3-AR expression correlates with the species-specific ability to face low oxygen (e.g. hypoxia-sensitive trout versus hypoxia-tolerant goldfish).

1.3.2. Effects of β3-AR stimulation in the normoxic goldfish heart

In the isolated goldfish heart perfused under normoxic conditions, β3-AR stimulation by BRL_{37344} induced a concentration-dependent increase of contractility. This effect was abolished by the β3-AR antagonist SR_{59230A}, which acts in a competitive manner in teleosts (Imbrogno, et al., 2015). In contrast, it was not modified by a mixture of α/β1/β2-AR antagonists (phentolamine, nadolol and ICI118,551, respectively), free of β3-ARs antagonist properties (Emorine, et al., 1989; Galitzky, et al., 1993). This excluded the involvement of these AR types in the hemodynamic effects elicited by β3-AR activation. In mammals, a promiscuous coupling of β3-ARs to either $G_{i/o}$ or G_s proteins has been proposed to describe the different cardiac effects so far reported (Imbrogno, et al., 2015; Sterin-Borda, et al., 2006). As originally proposed by Gauthier and colleagues (Gauthier, et al., 1996; Gauthier, et al., 1998), and then confirmed by several experimental evidences, cardiac β3-ARs are generally considered to be coupled to $G_{i/o}$ and to a transduction mechanism that, through the involvement of NO, leads to a reduction of Ca^{2+} transients (Kitamura, et al., 2000; Mazza, et al., 2010) and dampens the stimulatory effects induced by cAMP cascades. In parallel, several lines of evidence suggest a role for G_s proteins (Bardou, et al., 2000; Mattsson, et al., 2010; Zhang, et al., 2012). This is the case for the positive inotropism observed in the mouse heart after stimulation of β3-ARs that is associated with a G_s dependent activation of AC and the consequent cAMP generation (Kohout, et al., 2001). A G_s-induced increase of L-type Ca^{2+} channel current ($I_{Ca,L}$), through a cAMP/PKA mechanism, has been also used to

explain the increased contractility induced by β3-AR agonists SR_{58611}, BRL_{37344} and CG_{P12177} in human atrial myocytes (Skeberdis, et al., 2008). The mechanisms by which teleost β3-AR activation induces cardiac effects are largely unknown. In the eel, the reduction of contractility induced by BRL_{37344} was abolished by the pre-treatment with pertussis toxin (PTx), a toxin that uncouples the signal transduction between several families of receptors and Gi/o proteins (Imbrogno, et al., 2010), thus pointing to a mechanism of action that recruits PTx-sensitive G proteins. We show here that the treatment with the specific AC inhibitor, MDL-12,3330A, abolished the effects induced by β3-AR activation, suggesting a transduction pathway that, unlike the eel, appears mediated by G_s proteins and AC. This was corroborated by the rise of cAMP levels detected in BRL_{37344}-treated heart. Experiments are ongoing in our laboratory to identify the specific intracellular targets that in the goldfish heart are modulated downstream the β3-AR–AC–cAMP cascade.

1.3.3. Role of β3-ARs in the hypoxia-induced modulation of goldfish cardiac contractility

A remarkable feature of the goldfish heart exposed to acute hypoxia is its ability to enhance the basal cardiac performance and the sensitivity to the Frank–Starling mechanism (Imbrogno, et al., 2014). This has been considered to be an important mechanism for maintaining functional and metabolic interactions between organs and tissues, required for the hypoxia tolerance of the whole organism (Gattuso, et al., 2018). We observed that under acute administration of moderate hypoxia, β3-AR-selective inhibition by SR_{59230A} abolished the time-dependent improved cardiac performance of the hypoxic goldfish heart. This effect was not observed by administration of phentolamine (a non-selective α-AR antagonist), nadolol (a non-selective β-AR antagonist) and ICI118,551 (a selective β2-AR antagonist),

thus ruling out the involvement of α/β1/β2-ARs. Unlike normoxia, under hypoxia, β3-AR activation by BRL_{37344} did not affect goldfish heart performance. This result is intriguing but deserves further study to establish whether in the goldfish differences in oxygen availability affect myocardial β3-AR sensitivity. The study of the mechanism of action elicited by β3-AR activation showed that, as in normoxia, the AC–cAMP cascade is involved. This was suggested by the effect induced by the AC inhibitor MDL-12,3330A on the time-course experiments, and by the increased levels of cAMP we observed in the hypoxia-exposed hearts. Currently, the specific mechanism responsible for β3-AR activation in our isolated hypoxic goldfish hearts is unknown. Interestingly, as reported by Newton and co-workers, a rich adrenergic innervation, which may allow a fine autonomic control of the cardiac function, characterizes the goldfish heart (Newton, et al., 2014). Moreover, it has been proposed that the isolated and perfused goldfish heart is able to release CAs, possibly from intracardiac chromaffin tissues (Cameron & O'Connor, 1979), and that in the hypoxia-tolerant tropical fish red-bellied piranha (*Pygocentrus nattereri*), an endogenous release of cardiac CAs is able to rescue myocardial performance during hypoxia, even in the absence of humoral CAs (Joyce, et al., 2019). In this context, the possibility that in the goldfish heart, perfused under hypoxia, β3-ARs may be activated by CAs released by either endogenous chromaffin tissues and/or by nerve terminals, should be considered.

1.4. CONCLUSIONS

This study documented the expression of functional β3-ARs in the heart of the goldfish *C. auratus*. Activation of these receptors positively affected the cardiac contractile performance under normoxia and contributed to the time-dependent increase of contractility, which characterizes the hypoxic goldfish heart. Under both normoxia and hypoxia, these effects involve a cAMP-dependent transduction pathway. Our results enrich the knowledge on the basal adrenergic control of the goldfish heart and are the first to propose β3-ARs as components of the complex molecular machinery that, in this fish, allows hypoxia tolerance. Further investigations will hopefully contribute to better characterize the goldfish as a versatile experimental tool, to be regarded with interest also for translational studies aimed to decipher the mechanisms that in the heart may be activated to face conditions of low oxygen.

CHAPTER 2.

The hypoxia tolerance of the goldfish (Carassius auratus) heart: the NOS/NO system and beyond

2.1. INTRODUCTION

Since its discovery in the 1772, NO has been recognized as the major organizer of the complex cardiac intracellular signals. This gasotransmitter is generated by the family of NOS isoenzyme which include the constitutive endothelial (eNOS) and neuronal (nNOS), and the inducible (iNOS), isoforms. By using molecular oxygen and NADPH as essential cofactors, these enzymes convert L-arginine into L-citrulline and NO. This reaction, because of the obligatory requirement for molecular O_2, is vulnerable to hypoxia (Moncada & Higgs, 1993; Bryan, 2006; Lundberg, et al., 2008). NO exerts its physiological effects by reversible binding and/or reacting with hemes, thiols or amines, forming iron-nitrosyl (FeNO), S-nitroso (SNO) and N-nitroso (NNO) compounds (Hill, et al., 2010). NO has a very short half-life. It is rapidly metabolized to nitrite in reaction with O_2 (Lundberg, et al., 2008), and is inactivated by oxidation to nitrate in reaction with oxygenated hemoglobin (Hb) and myoglobin (Mb). It can also react with the superoxide anion to produce peroxynitrite ($ONOO^-$) (Ronson, et al., 1999), a highly reactive product (Guzik, et al., 2002) able to form additional reactive nitrogen species (RNS). All RNS are responsible of protein post-translational modifications through either S-nitrosation [i.e., the formation of a covalent bound between a NO^+ equivalent and a nucleophilic center (amine or thiol)], or S-nitrosylation [i.e., the addition of NO to a reactant without changing the formal charge of the substrate (metal centers or radical species)] (Heinrich, et al., 2013). Under hypoxia, NO may also determine protein nitration. This consists in the substitution, mainly under the action of $ONOO^-$, of a nitro group to tyrosine residues, to give 3 nitrotyrosine (3-NT) (Radi, 2004). Uncontrolled nitrosation/nitrosylation/nitration cause nitrosative stress, responsible in turn for changes in protein activity, stability, conformation and/or interaction with other

molecules (Foster, et al., 2009). It has been revealed that, when the NOS activity is compromised by low O_2, an increased NOS expression and/or a nitrite/nitrate conversion to NO stabilize NO levels, providing an alternative pathway for gas generation (Imbrogno, et al., 2014; Hansen & Jensen, 2010; Sandvik, et al., 2012; Hansen, et al., 2016). This NO regeneration may involve Xanthine Oxidoreductase (XOR), mitochondrial enzymes and deoxygenated Hb, Mb and neuroglobin (Hansen & Jensen, 2010). The nitrate-nitrite-NO pathway is complementary to the classical L-arginine-NOS pathway, and becomes predominant in response to a reduced O_2 availability.

In mammals, the NOS/NO system has been proposed as a convergent knot of many regulatory and neuroendocrine pathways involved in the control of heart function. Particularly, the activation of this system has been correlated to the mechanisms which maintain cardiac health under low oxygen. A hypoxia-dependent increase of NO helps mammalian myocardial cells to limit cardiac injury caused by low O_2 supply. Of note, in the presence of ischemia, the NO dependent suppression of the electron-transport chain, by reducing mitochondrial energy production, limits the cardiac damages induced by ischemia/reperfusion (IR) (Shiva, et al., 2007).

The crucial role exerted by NO during hypoxia has been recently extended also to non-mammalian vertebrates, including fish. A growing body of evidence has documented the cardioprotective role of NO and its related nitrosative signal under hypoxic challenges. This information mainly derives from studies on the goldfish *C. auratus*. It has been reported that in the goldfish, NO inhibits mitochondrial respiration without affecting contractility (Pedersen, et al., 2010). This increases myocardial efficiency (i.e., the force generated per O_2 consumed), thus contributing to maintain fish myocardial function in the presence of

hypoxia or anoxia (Stecyk, et al., 2004a). It has been also reported that in the goldfish heart, the hypoxia-induced increase in NO production could activate sarcolemmal KATP channels, a response that may enhance hypoxia tolerance (Cameron, et al., 2003). Of note, in the hypoxic goldfish heart, the increased NOS expression is accompanied by an enhanced expression of HIF-1 α responsible, in turn, for the activation of genes critical for cell survival (Ong & Haunseloy, 2012). However, so far, limited pieces of evidence are available concerning the intracellular signals activated by NO during hypoxia.

Moved by these premises, and by taking advantage of the goldfish *C. auratus* as a natural model of hypoxia resistance, we furnished a deeper insight into the NO targets and downstream events activated in cardiac cells under low O_2.

2.2. RESULTS

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2.2.1. Isolated heart preparations

The *ex vivo* isolated and perfused goldfish heart showed basal values of preload, afterload, HR, CO, SV and SW (Table 2) comparable to those previously reported [normoxia: (Imbrogno & Cerra, 2017; Mazza, et al., 2015); hypoxia: (Imbrogno, et al., 2014)].

Table 2. *Baseline cardiac parameters of the isolated goldfish (Carassius auratus) heart, perfused under either normoxia or hypoxia; Values are means ± s.e.m. of n=9 (normoxia) and n=13 (hypoxia) experiments*

	CO (ml min^{-1} kg^{-1})	**SV** (ml kg^{-1})	**HR** (beats min^{-1})	**SW** (mJ g^{-1})	**Preload** (kPa)	**Afterload** (kPa)
Normoxia	13.531±0.379	0.186±0.016	76.333±5.459	0.235±0.020	0.073±0.002	1.413±0.018
Hypoxia	13.777±0.479	0.194±0.017	76.769±6.299	0.288±0.032	0.064±0.006	1.434±0.031

2.2.2. Role of the NOS/NO system in the hypoxia-induced increase of contractility

Exposure of the isolated and perfused goldfish heart to a hypoxic medium is accompanied by a time-dependent enhancement of the mechanical performance (Imbrogno, et al., 2014). To evaluate whether the NOS/NO system is involved in the hypoxia-induced cardiac stimulation, time-course experiments were performed in the presence of the NOS inhibitor

L-NMMA (10^{-5} mol l^{-1}), or the NO scavenger PTIO (10^{-6} mol l^{-1}). Both treatments abolished the hypoxia-dependent increase of contractility (Figure 7), strongly supporting a NOS-produced NO contribution.

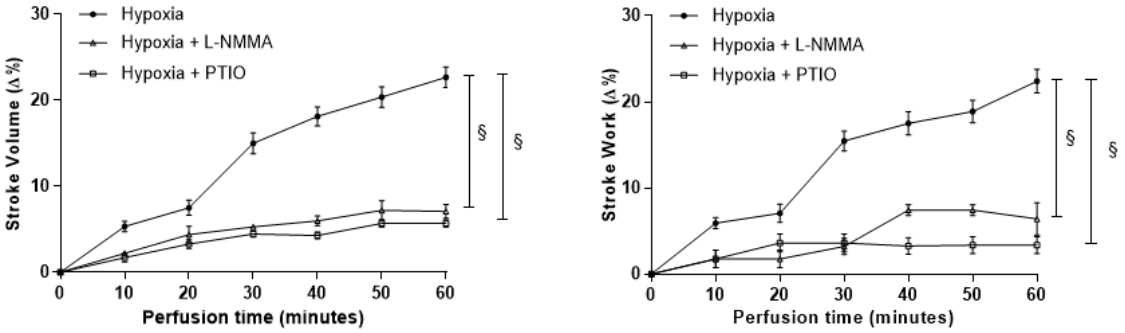

Figure 7. *Time-course curves for the Stroke Volume (SV) and Stroke Work (SW) of the isolated and perfused goldfish heart before and after treatment with either L-NMMA (10^{-5} mol l^{-1}) or PTIO (10^{-6} mol l^{-1}). Data are expressed as mean values ± s.e.m. of 4/7 experiments for each group. Statistics were assessed by one-way ANOVA followed by Bonferroni's post hoc test (§$p < 0.05$; hypoxia versus hypoxia plus either L-NMMA or PTIO)*

2.2.3. PI3-K/Akt-dependent NOS activation

The PI3-K/Akt pathway plays a relevant role in the NOS activation and the subsequent NO production [for references in fish see (Garofalo, et al., 2009)]. To verify its putative involvement in the hypoxia-induced increase of contractility, the response of the goldfish heart to hypoxia was evaluated before and after treatment with Wortmannin (10^{-9} mol l^{-1}), a PI3-K inhibitor. As indicated in Figure 8A, Wortmannin abolished the increase of SV and SW, suggesting a mechanism that, *via* a PI3-K-dependent pathway, induces the NOS/NO system activation. Consistent with this, cardiac homogenates from goldfish hearts perfused under hypoxia showed significant increased levels of the phosphorylated form of the NOS controlling protein Akt (pAkt), as evidenced by western blotting and densitometric analysis (Figure 8B). After treatment with Wortmannin, pAkt values returned to levels comparable to those detected under normoxia (Figure 8B).

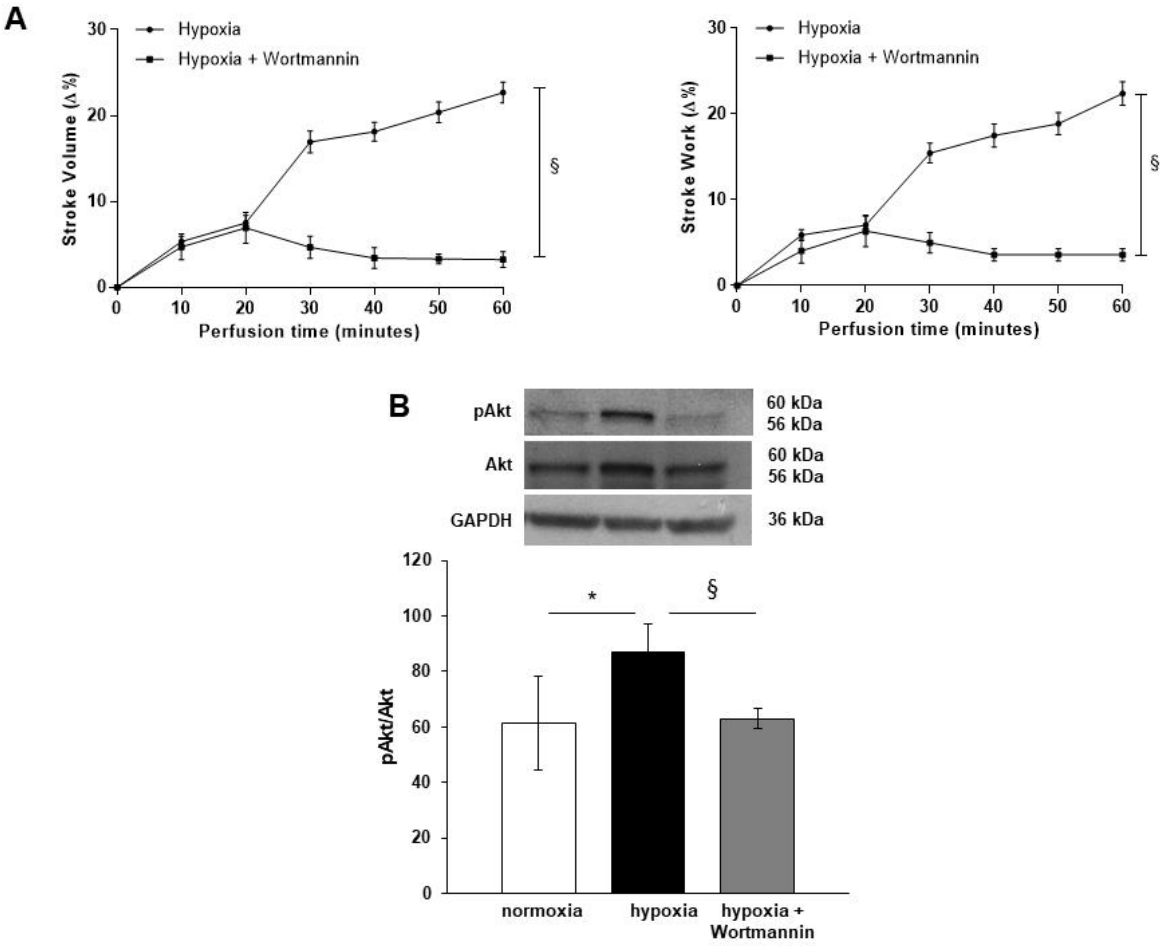

Figure 8. (A) *Time-course curves for the Stroke Volume (SV) and Stroke Work (SW) of the isolated and perfused goldfish heart before and after treatment with Wortmannin (10^{-9} mol l^{-1}). Data are expressed as mean values ± s.e.m. of 4 experiments. Statistics were assessed by two-tailed unpaired t-test (§p< 0.05; hypoxia versus hypoxia plus Wortmannin).* ***(B)*** *Representative Western Blotting and densitometric analysis of pAkt (Ser473)/Akt expression in goldfish cardiac extracts under normoxia, hypoxia and hypoxia plus Wortmannin. Data were expressed as means ± s.e.m. of absolute values from individual experiments (n=3); statistics were assessed by two-tailed unpaired t-test (*p< 0.05)*

2.2.4. NO intracellular signals

2.2.4.1 Role of cGMP

NO exerts an important modulation of the cardiac performance *via* a cGMP-dependent pathway activation (Imbrogno, et al., 2010; Angelone, et al., 2012; Imbrogno, et al., 2001). To assess if in the hypoxic goldfish heart, the time-dependent increase of contractility involves cGMP generation, cGMP levels were measured in homogenates of hearts perfused under

either normoxia or hypoxia. Results showed no differences between the two conditions (Figure 9), thus excluding the involvement of this second messenger in the mechanisms whereby NO modulates the goldfish heart response to hypoxia.

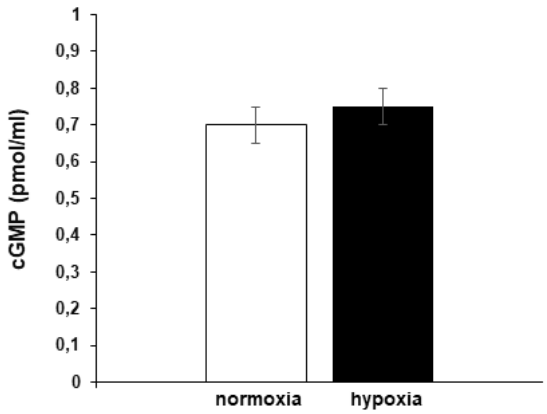

***Figure 9**. cGMP levels in goldfish cardiac extracts under normoxia and hypoxia. Data were expressed as means ± s.e.m. of absolute values from individual experiments (n=3); statistic was assessed by two-tailed unpaired t-test (*p<0.05).*

2.2.4.2. Analysis of S-nitrosylated proteins

S-nitrosylation, the covalent modification of a protein cysteine thiol by a NO group to generate a S-nitrosothiol (SNO), represents a cGMP-independent mechanism modulating many physiological pathways (Garofalo, et al., 2009; Rossi-George & Gow, 2013).

To assess, in the goldfish heart, the pattern of proteins containing S-nitrosylated cysteines, the Biotin Switch assay was used on homogenates of control hearts and of hearts exposed to hypoxia. With respect to the normoxic counterpart, cardiac tissues exposed to hypoxia showed a significant reduction of S-nitrosylation of a broad range of proteins (Figure 10A and B).

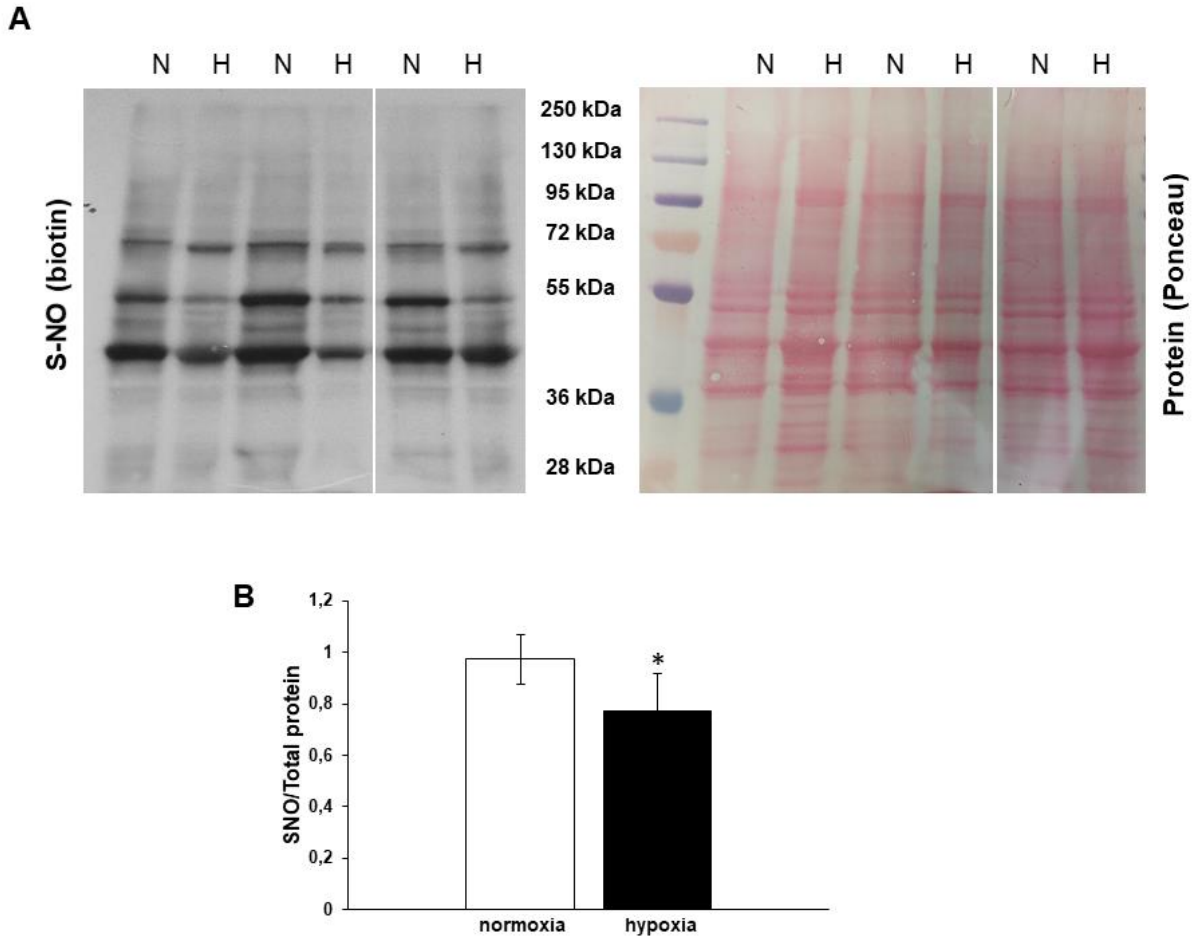

Figure 10. ***(A)*** *Blots and corresponding Ponceau staining of s-nitrosylated proteins in homogenates of goldfish hearts perfused under either normoxia (N) or hypoxia (H).* ***(B)*** *Densitometric analysis of normalized S-NO (biotin)/Ponceau signal. Data were expressed as means ± s.e.m. of absolute values from individual experiments (n=3); statistics were assessed by two-tailed unpaired t-test (*p < 0.05)*

2.2.4.3. Role of SERCA2a pumps

Evidence in mammals designed NO as a key modulator of Ca^{2+} cycling, influencing Ca^{2+} channels and SERCA2a pumps (Casadei & Sears, 2003; Sears, et al., 2003). In fish, a regulatory role of NO on cardiac calcium reuptake by SERCA2a emerged in the eel (*Anguilla anguilla*) (Garofalo, et al., 2009). In the goldfish heart, the role of SERCA2a pumps in the response to hypoxia was evaluated by exposing isolated heart preparations to hypoxia in the presence of the specific inhibitor Thapsigargin (10^{-7} mol l^{-1}). This inhibitor significantly

reduced the time-course increase of contractility (Figure 11), suggesting that the nitrergic modulation of the goldfish heart in response to low O_2 availability occurs *via* a NO-dependent modulation of the rate of Ca^{2+} re-uptake by SR.

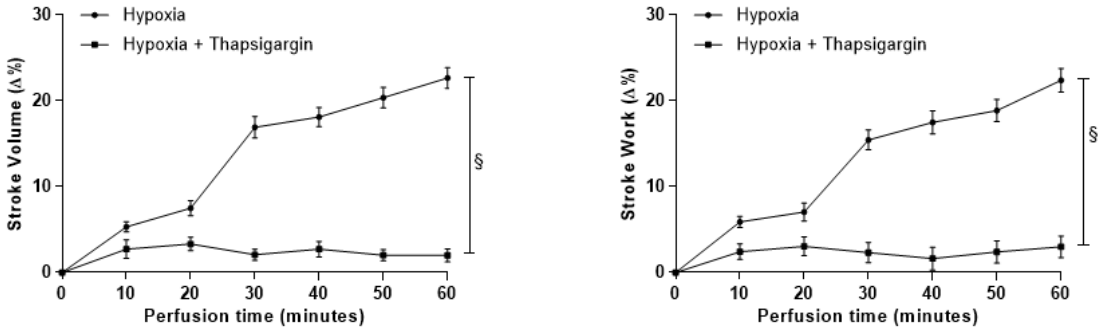

Figure 11. *Time-course curves of Stroke Volume (SV) and Stroke Work (SW) of the isolated and perfused goldfish heart before and after treatment with Thapsigargin (10^{-7} mol l-1). Data are expressed as mean values ± s.e.m. of 4 experiments. Statistic was assessed by two-tailed unpaired t-test (§$p<0.05$; hypoxia vs hypoxia plus Thapsigargin).*

2.2.5. Nox2 expression

NADPH oxidase represents one of the major cellular sources of O_2^-. In the heart, it is involved in many physiological and pathological processes, including hypoxic adaptation (Brandes, et al., 2010). To investigate whether, in the goldfish heart, hypoxia can influence NADPH oxidase activity, the expression levels of Nox2, the catalytic subunit of the enzyme, were investigated by western blotting. As shown in Figure 12, an immunoreactive band corresponding to the predicted molecular weight of Nox2 was detected in homogenates of hearts perfused under either normoxic or hypoxic conditions. In particular, the resulting Nox2 expression was significantly increased in goldfish hearts exposed to hypoxia.

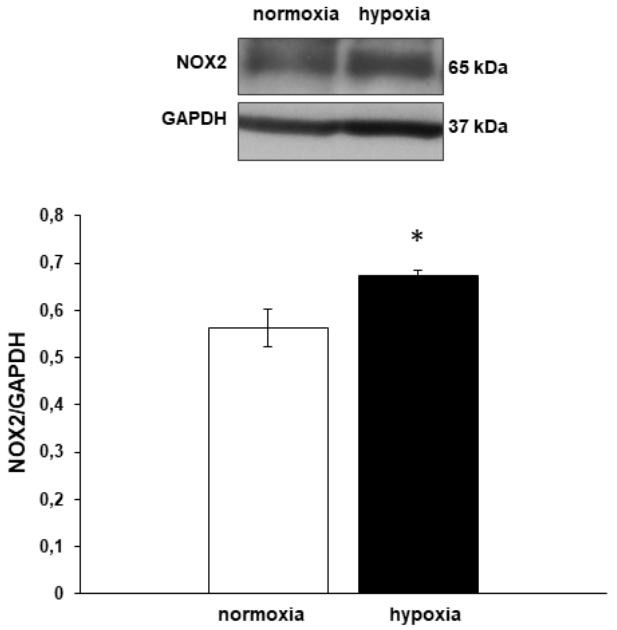

***Figure 12**. Western blotting and densitometric analysis of Nox2 (gp91phox) expression in extracts of goldfish heart perfused under normoxic and hypoxic conditions. Data were expressed as means ± s.e.m. of absolute values from individual experiments (n=3); statistic was assessed by two-tailed unpaired t-test (*p<0.05).*

2.3. DISCUSSION

By using the goldfish as a gold standard of hypoxia tolerance, we explored whether the NOS/NO system and the downstream-activated signals provide advantage to the heart under low O2. To the best of our knowledge, our data are the first to show that NO sustains the intense contractility of the hypoxic goldfish heart via a mechanism which is independent of cGMP, and involves a PI3-K/Akt-mediated activation of NOS-dependent NO production and SERCA2a pumps. The denitrosylation and/or putative nitration of intracellular targets have also been evaluated as related mechanisms that contribute to the high resistance of the goldfish heart to hypoxia.

2.3.1. PI3-K/Akt/NOS/NO pathway activation

The remarkable ability of the goldfish heart to enhance its basal performance when exposed to a hypoxic milieu has been largely documented by studies from our laboratory (Imbrogno, et al., 2014; Leo, et al., 2019). These studies reported that in *C. auratus*, exposure to hypoxia is accompanied by an increase of cardiac HIF-1α (hypoxia-inducible factor 1α) and NOS expression, expanding to this teleost the protective role elicited by NO to the hypoxic myocardium (Imbrogno, et al., 2014), already proposed in mammalian and non-mammalian vertebrates [see for references (Fago & Jensen, 2015; Tota, et al., 2011)]. In agreement with these results, we now observed a significant reduction of the hypoxia-dependent increase of contractility in hearts perfused in the presence of the NO scavenger PTIO or the NOS inhibitor L-NMMA, which represents the physiological evidence of the NO need for the hypoxic goldfish heart. This was confirmed by the hypoxia-induced activation of the PI3K/Akt pathway, a well-known player in the NOS-dependent NO production (see for example (Yu, et al., 2011; Carrillo-Sepulveda, et al., 2010), as documented by the significant

reduction of contractility induced by the PI3-K inhibitor Wortmannin, and by the increased level of pAkt in hearts perfused under hypoxia. In line with this, extracts from hypoxic hearts treated with Wortmannin showed pAkt levels similar to those detected under normoxia. In mammals, Akt represents not only the effector of the PI3-K-mediated NOS activation pathway, but also a cardioprotective factor, able to regulate a variety of cellular functions under hypoxia (Chaanine & Hajjar, 2011). In the ischaemic mammalian heart, Akt promotes the utilization of glucose, instead of toxic free fatty acids, and the efficient consumption of oxygen by the myocardium (Chaanine & Hajjar, 2011). Moreover, in response to hypoxia, adenoviral gene transfer of activated Akt protects cardiomyocytes from apoptosis (Matsui, et al., 1999). Akt may also directly improve the contractile function of the myocardium by increasing SERCA2a levels, or by enhancing its activity through phosphorylation, and thus inhibition, of phospholamban (PLN) (Chaanine & Hajjar, 2011). Studies in non-mammalian vertebrates show that Akt plays a role in the cardiac response to environmental, physical and chemical stimuli [eel: (Garofalo, et al., 2009); lungfish: (Amelio, et al., 2013); frog: (Mazza, et al., 2010; Mazza, et al., 2013)]. In addition, in the hypoxic goldfish heart, Akt has been proposed to mediate the effects elicited by Selenoprotein T-derived peptide (see CHAPTER 3), a cardioprotective factor which in mammals reduces ischemia–reperfusion injury (Rocca, et al., 2018). In line with these observations, our results strongly support the possibility that Akt, by acting in concert with other cardioprotective factors, may contribute to the hypoxia resistance of the goldfish heart.

2.3.2. NO downstream effectors

A complex chemistry and target factors are involved in the NO-mediated intracellular effects. Under hypoxia, this picture is further complicated by the presence of reactive oxygen species and their connection with NO-related products (Figure 13).

Aiming to disentangling the mechanisms activated in the hypoxic goldfish heart downstream NO production, we analysed the involvement of cGMP, the classic cardiac NO mediator. In the working goldfish heart, the NO-induced cGMP generation remarkably modulates mechanical performance, tonically decreasing SV under basal conditions (Imbrogno, et al., 2014). However, data obtained in the present study excluded the involvement of cGMP in the mechanisms responsible of the time-dependent increase of myocardial contractility experienced by goldfish under low O_2, as indicated by the comparable levels of cGMP detected by ELISA test in homogenates of hearts perfused under either normoxia or hypoxia.

Accumulating evidence indicate that major NO-mediated non-cGMP signals are those related to the covalent attachment of NO to the thiol group of cysteine (Cys) residues (S-nitrosylation) (Hess et al., 2005). In fish, the pattern of S-nitrosylated proteins was studied by our research group through biotin switch assay in eel (*A. anguilla*) cardiac tissues in response to both nitrite (Cerra, et al., 2009)and preload (Garofalo et al., 2009) stimulation, showing an increase of protein S-nitrosylation. By using the same experimental approach, we now found a significant reduction in the degree of S-nitrosylated proteins in goldfish hearts exposed to hypoxia with respect to the normoxic counterpart. To date, about 3,000 proteins have been identified as targets of S-nitrosylation (Rizza, et al., 2019), indicating the potential significance of a controlled mechanism for a proper cardiac function. Accordingly,

dysregulated protein S-nitrosylation has been correlated with the etiology of several heart, muscle, and lung diseases, as well as of cancer and neurodegenerative disorders (Durham, et al., 2008; Nakamura, et al., 2006). By using transgenic mouse overexpressing in cardiomyocytes the denitrosylating enzyme S-nitrosylated glutathione reductase (GSNOR), Sips and co-workers proposed protein denitrosylation as a protective mechanism against the development of myocardial dysfunction under stress conditions (Sips, et al., 2013). Works are in progress in our laboratory to identify proteins encountering denitrosylation in the hypoxic goldfish heart, and the related functional significance. Waiting for more detailed information, and in agreement with the mammalian data, the present results propose denitrosylation as a mechanism that is activated in fish under conditions of hypoxic stress to sustain cardioprotective programs.

It is known that NO forms RNS in the presence of excess reactive oxygen species. In particular, the fast reaction of NO with superoxide (O_2^-) leads to the formation of peroxynitrite ($ONOO^-$) (Figure 13), which in turn may affect different intracellular biomolecules (Bartesaghi, et al., 2011). An important aspect of peroxynitrite-mediated protein modification is its ability to promote tyrosine nitration, the substitution of a hydrogen by a nitro group in the position 3 of the phenolic ring, generating 3-NT. Functional studies of nitrated proteins indicate that nitrotyrosine modifications alter protein catalysis, protein–protein interaction, and tyrosine kinase signalling (Ischiropoulos, 2003). However, rather than inducing protein damage, nitration is proposed as a control mechanism of redox homeostasis in normally functioning cardiac muscle (Bigelow, 2009). Interestingly, by western blotting analysis, we observed an increase of Nox2 expression, indicative of an increased NADPH oxidase activity, in the highly performing hypoxic goldfish heart. These

evidences, which agree with the increased (but not detrimental) levels of 3-NT we previously observed in goldfish hearts exposed to hypoxia (see CHAPTER 3), support the possibility that nitration may contribute to the high resistance of the goldfish heart to conditions of reduced oxygen.

Tyrosine nitration is a highly selective process, since neither all proteins can be nitrated nor all tyrosine residues within a particular protein can undergo nitration. At this purpose, it has been reported that in whole tissue/cell only 1–5 over 10,000 tyrosine residues may be nitrated (Radi, 2004). However, in several specific proteins, nitrated tyrosine residues are numerous, and this is responsible of important protein structural and functional changes (Radi, 2004; Batthyany, et al., 2017; Radi, 2013). In this context, our data are of interest since they can provide a conceptual basis to explore and clarify the apparent contradiction between the established benefits of NO supplementation under hypoxia, and the general concept of RNS, and related downstream-activated cascades, as deleterious to the cells.

An important intracellular target of NO is represented by the SERCA [see for example (Cerra & Imbrogno, 2012; Adachi, et al., 2004)], the integral membrane protein controlling Ca^{2+} homeostasis through its active transport across the sarcoplasmic reticulum. SERCA2a is the predominant isoform in cardiac muscle. By ensuring sufficient Ca^{2+} load in the SR, it modulates muscle relaxation as well as contraction (Misquitta, et al., 1999; Periasamy & Huke, 2001; Periasamy & Kalyanasundaram, 2007). SERCA2a is regulated by PLN, a 52 amino acid protein that, in its de-phosphorylated state, binds to the pump decreasing its affinity for Ca^{2+} (Frank & Kranias, 2000; MacLennan & Kranias, 2003). When phosphorylated, PLN dissociates from SERCA2a, thus restoring its affinity for Ca^{2+} (MacLennan & Kranias, 2003). In addition to PLN, SERCA2a pumps are highly susceptible

to oxidative and nitrosative modifications, as they contain vulnerable cysteine and tyrosine residues [see for ref (Bigelow, 2009; Braun, et al., 2019)]. Nitrotyrosine modification of SERCA2a has been observed in several pathophysiological conditions (Viner, et al., 1996) and nitrated SERCA2a has been recently considered as a cardiac marker of nitrative stress (Bigelow, 2009). It has been proposed that the close structural proximity between mitochondria and SERCA2a pumps exposes SERCA2a to reactive oxygen/nitrogen species which derive from the superoxide generated as a by-product of mitochondrial oxidative phosphorylation (Cadenas, 2004), also providing a mean to regulate energy metabolism under conditions of stress (Bigelow, 2009).

The nitration of specific proteins, such as the SERCA2a pump, was not specifically assessed by the present study; however, the abolition of the hypoxia-dependent increase of the cardiac performance induced by the SERCA2a specific inhibitor Thapsigargin clearly suggests a mechanism which involves SERCA2a-controlled muscle relaxation. Interestingly, in fish, the aminoacid sequence of SERCA2a (zebrafish: NP_957259.1) includes tyrosine residues (i.e., 294-295 and 753) which in mammals are recognized as potential site of nitration. This opens another suggestive route for investigations.

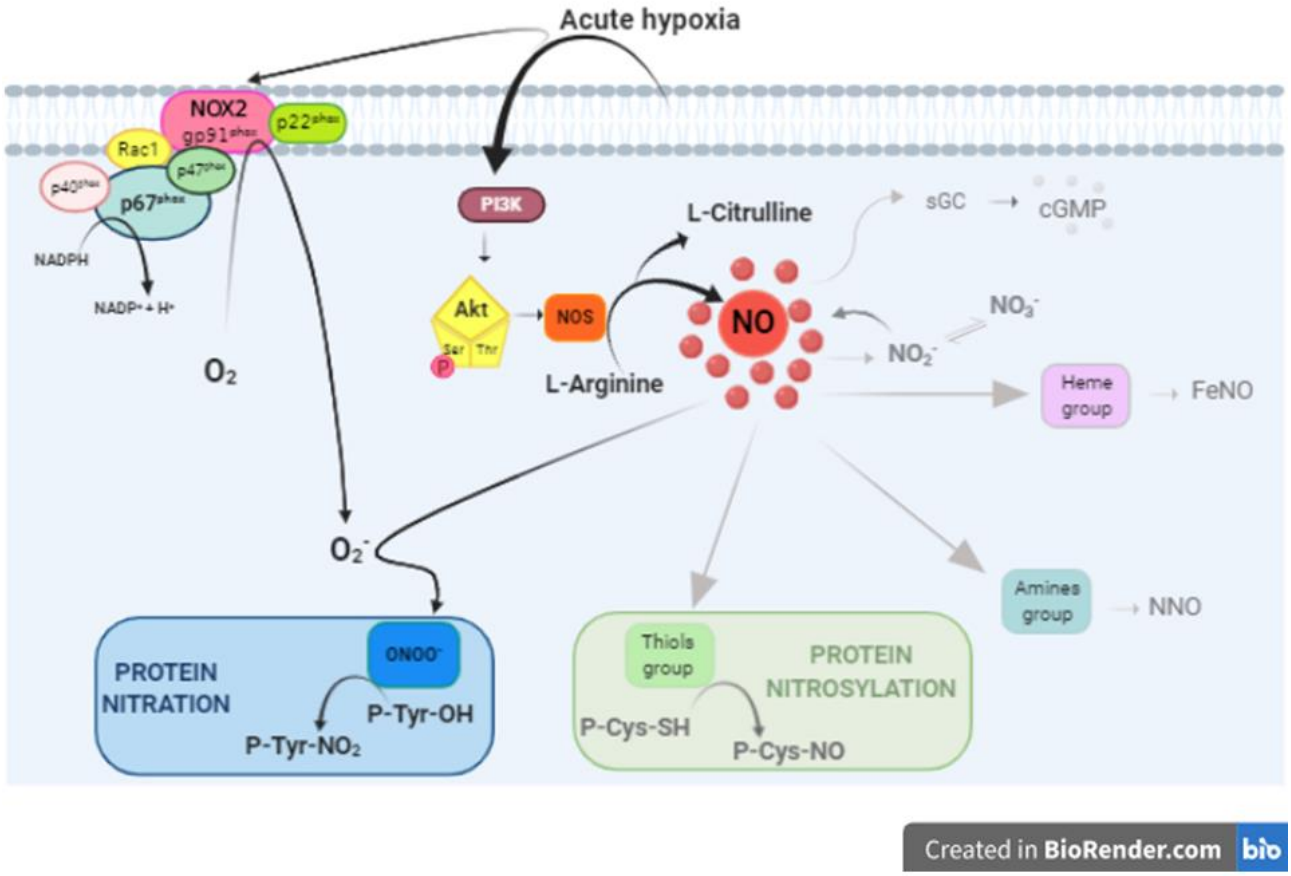

Figure 13. *Simplified overview of NO-mediated intracellular pathways. NO may activate the soluble guanylyl cyclase (sGC) to produce cGMP, or interact with heme, amines or thiols to produce iron nitrosyl (FeNO), s-nitroso (SNO) and n-nitroso (NNO) compounds. On the other hand, NO can react with superoxide radicals (O_2^-) to generate peroxynitrite ($ONOO^-$). Our results suggest that under acute hypoxia, the activation of the PI3-K/Akt pathway and the Nox2 enzyme may cause the simultaneous generation of NO and O_2^-, respectively, thus contributing to protein nitration (black arrows).*

2.4. CONCLUSIONS

In conclusion, the proposed study revealed novel aspects of the still unresolved mechanisms that sustain the elevated hypoxic tolerance of the goldfish heart, making this teleost a precious animal model. We showed the involvement of a PI3-K/Akt/NOS/NO cascade that escapes the classic cGMP generation, but is paralleled by SERCA2a pumps activation and increased expression of Nox2. Remarkably, for the first time, protein S-denitrosylation was found to be associated to the exposure of the goldfish heart to low O_2. A dynamic balance between nitrosylation and denitrosylation of proteins is critical for a proper myocardial functioning, also in response to stress (Viner, et al., 1996). Further studies will clarify the significance of denitrosylation in the goldfish heart challenged by hypoxia, for example by identifying the specific proteins that undergo denitrosylation. Another point to be resolved will be the apparent contradiction between NO generation and denitrosylation. As we suggested, NO-dependent protein nitration may represent a concurrent phenomenon that enhances the spectrum of opportunities for NO to protect the stressed goldfish heart.

CHAPTER 3.

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

3.1. INTRODUCTION

Selenoproteins are a widely distributed family of proteins containing selenium in the form of the 21st amino acid selenocysteine (Sec) which play important roles in maintaining the cellular redox balance (Ferguson, et al., 2006). 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). There are currently 25 known selenoproteins in the human proteome (Kryukov, et al., 2003), classified in 3 subfamilies including thioredoxin reductase, glutathione peroxidase, and iodothyroninedeiodinases. In 1999, by sing a bioinformatic algorithm that allows for the recognition of the SECIS element, Kryukov and colleagues (Kryukov, et al., 1999) identified a selenoprotein, named SelenoproteinT (SELENOT). Because of containing the thioredoxin-like fold and a conserved CxxU motif (where U represents Sec), SELENOT has been identified as a member of the thioredoxin-like protein family (Dikiy, et al., 2007). Within the cell, it mainly localizes in the endoplasmic reticulum (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 crucial 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 upregulated by the neurotrophic factor pituitary adenylate cyclase activating polypeptide (PACAP) during neuroendocrine differentiation (Grumolato, et al., 2003). 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., 2018). This involves the protective RISK (reperfusion injury salvage kinase) cascade and a reduction of apoptosis, and oxidative and nitrosative stress (Rocca, et al., 2018).

In fish, limited studies have analyzed the role of selenoproteins (Deniziak, et al., 2007; Mariotti, et al., 2012; Pacitti, et al., 2014). SELENOT paralogs have been identified in zebrafish (Kryukov & Gladyshev, 2000; Thisse, et al., 2003), as well as in the adult goldfish *C. auratus* in which they are designated as *gfSelT1a, gfSelT1b and gfSelT2* (Chen, et al., 2017). They present the SECIS element in the 3'-UTRs mRNA and the CxxU motif, typical of the thioredoxin-like proteins, in the N-terminal (Chen, et al., 2017). Constitutive expressions of *gfSelT1a, gfSelT1b and gfSelT2* were observed in several tissues, including the heart. Variation in mRNA expression of *gfSelT1a, gfSelT1b and gfSelT2* has been observed in response to environmental stressors such as cadmium exposure, oxidative stress and heat stress, suggesting an involvement in host protection against environmental stressors (Chen, et al., 2017). In particular, SELENOT could play a critical role in protecting the host from H_2O_2-mediated oxidative stress. This is consistent with the general pattern of increased antioxidant enzymes activity described in many aquatic organisms to reduce injury-related oxidative stress (Welker, et al., 2013). This primarily represents a putative mechanism of protection during hypoxia/anoxia reoxygenation, when the formation of ROS significantly increases. Since goldfish experiences hypoxia during its life cycle, a modulation of SELENOT expression in tissues more affected by oxygen deprivation, as the heart, can elicit

cytoprotection that contributes to finely regulate hypoxia activated mechanisms. Moved by this premises, we analysed the basal cardiac expression of SELENOT and the effects of exogenous PSELT on the performance of the *ex vivo* isolated and perfused working heart; the influences of acute hypoxia on the cardiac expression of SELENOT and the response of the goldfish heart to PSELT have been also investigated.

3.2. RESULTS

***Published in**: J. Exp. Biol. (2019), 222. Mazza, R.; Gattuso, A.; Imbrogno, S.; Boukhzar, L.; **Leo, S.;** Mallouki, B.Y.; Filice, M.; Rocca, C.; Angelone, T.; Anouar, Y.; Cerra, MC. Selenoprotein T as a new positive inotrope in the goldfish, Carassius auratus*

3.2.1. SELENOT expression in the fish heart

Basal SELENOT expression was evaluated in homogenates from non-perfused heart of juvenile and adult goldfish (juvenile: body length 3–5 cm, body weight 3.6±0.31 g mean±s.e.m.; adult: body length 12–16 cm, body weight 53.4±2.6 g). 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 (Figure 14A). A significantly lower expression was observed in the heart from adult fish with respect to the juvenile counterpart (Figure 14A). 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 (*Cyprinus carpio* Linnaeus 1758; body weight: 290±15 g), eel (*Anguilla anguilla* Linnaeus 1758; body weight: 95±6 g), zebrafish (*Danio rerio* F. Hamilton 1822; body weight: 0.61±0.04 g), and brown trout (*Salmo trutta* Linnaeus 1758; body weight: 210±12 g). Densitometric analysis showed that SELENOT was expressed in the cardiac extracts of all fish species examined (Figure 14B).

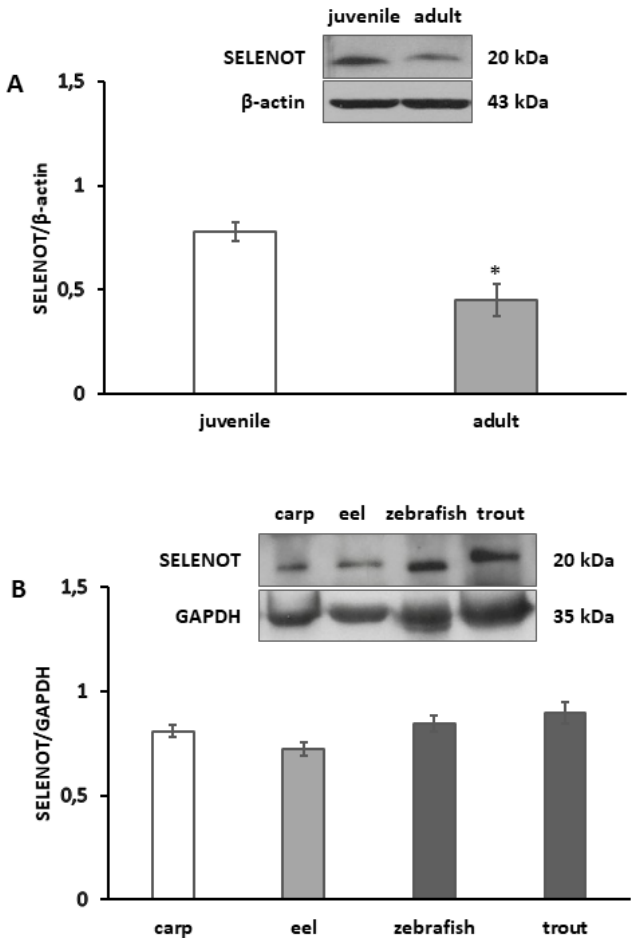

Figure 14. (A) *SELENOT expression (relative to that of β-actin) in non-perfused goldfish (Carassius auratus) hearts from juvenile (n=3) and adult fish (n=3). Significance of difference from control values (unpaired t-test): *p<0.05. Values are means ± s.e.m. of three individual experiments.* ***(B)*** *SELENOT expression (relative to that of GAPDH) in adult carp (Cyprinus carpio), eel (Anguilla anguilla), zebrafish (Danio rerio) and trout (Salmo trutta) cardiac extracts.*

3.2.2. Isolated heart preparation

After stabilization, the *ex vivo* isolated and perfused goldfish heart preparation values of preload, afterload, HR, CO, SV and SW (Table 3) were comparable to those previously reported [normoxia: (Garofalo, et al., 2012); hypoxia: (Imbrogno, et al., 2014)].

Table 3. *Baseline cardiac parameters of the isolated goldfish (Carassius auratus) heart, perfused under either normoxia or hypoxia; Values are means ± s.e.m. of n=29 (normoxia) and n=9 (hypoxia) experiments*

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

3.2.3. 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 SV, CO and SW at all concentrations tested (Figure 15A–C), thus acting as a positive inotropic agent. The enhancement of myocardial contractility occurred without modifying HR (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 (Figure 15D-F).

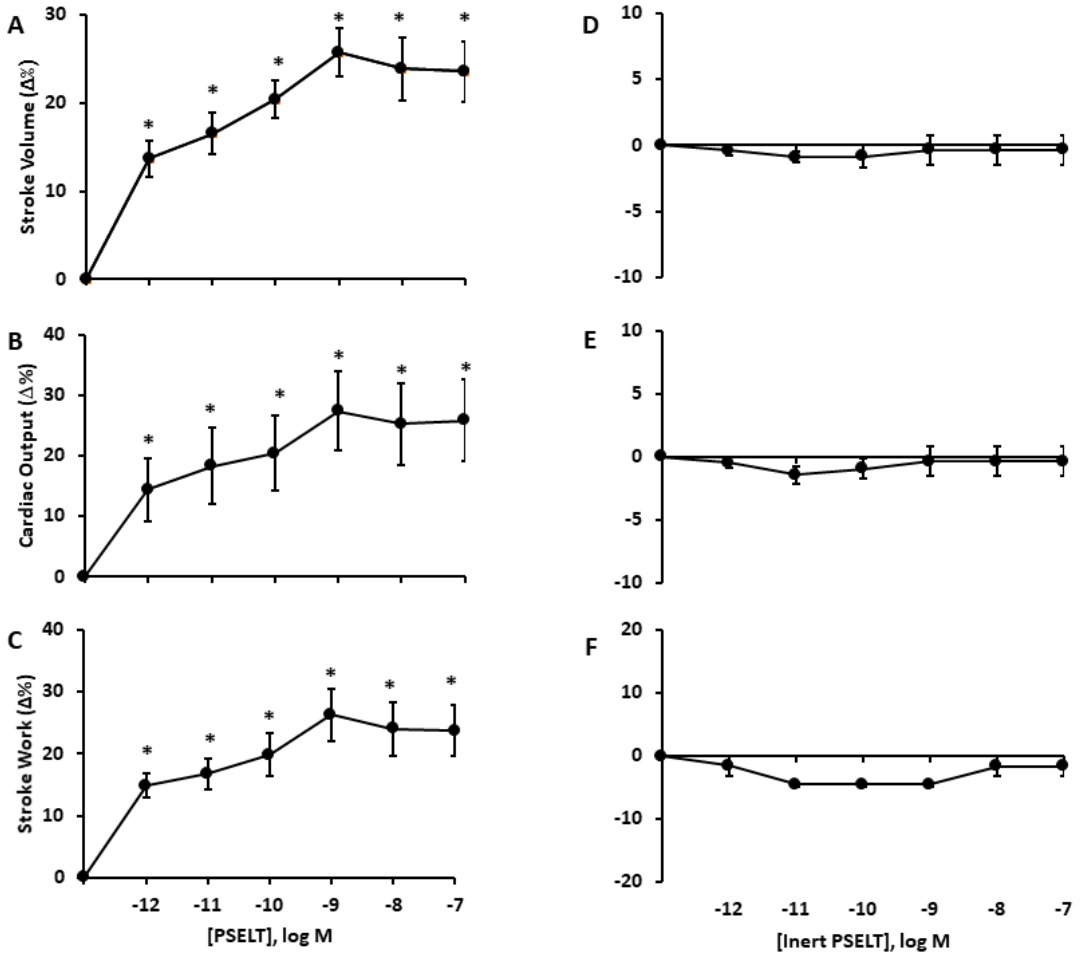

Figure 15. *Cumulative concentration–response curves for the effect of PSELT (left) and inert PSELT (right) on stroke volume, cardiac output and stroke work in the isolated working goldfish heart perfused under normoxia. Percentage change was evaluated as the mean ± s.e.m. of five experiments and is 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*

3.2.4. 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 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 (Figure 16 A). 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 Thapsigargin (10^{-7} mol l^{-1}). Both treatments abolished the stimulation of myocardial contractility induced by PSELT in the goldfish heart (Figure 16B), implicating a Ca^{2+}-dependent mechanism. The involvement of KATP 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 nonselective antagonist of KATP channels, or 5-HD (10^{-7} mol l^{-1}), a selective antagonist of mitochondrial KATP channels. Neither inhibitor modified the cardiac effects induced by PSELT (Figure 16C). 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) (Figure 17).

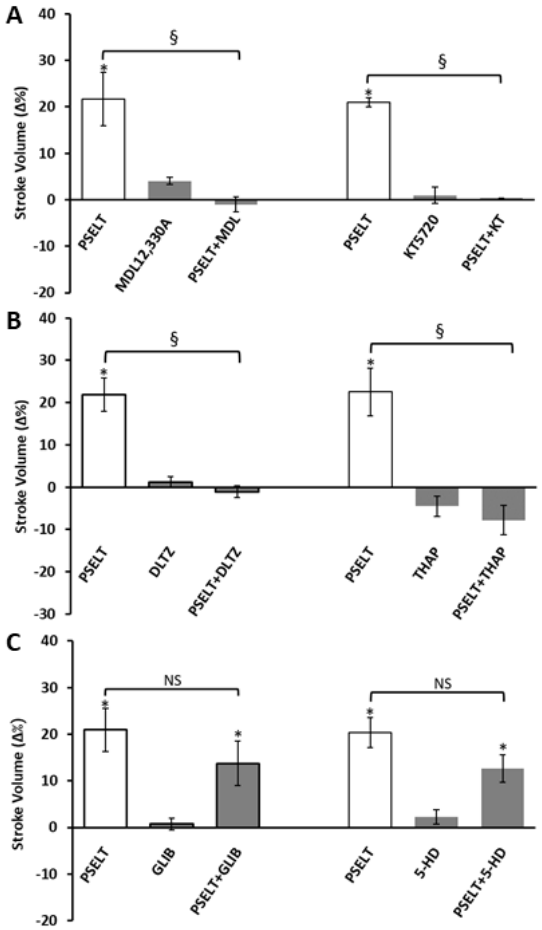

Figure 16. *Effect of PSELT (10^{-10} mol l^{-1}) before and after treatment with inhibitors on SV of the isolated and perfused working goldfish heart.* ***(A)*** *Adenylate cyclase inhibitor MDL123330A (MDL, 10^{-8} mol l^{-1}) and cAMP dependent kinase inhibitor KT5720 (KT, 10^{-7} mol l^{-1}).* ***(B)*** *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 KATP channel inhibitor glibenclamide (Glib, 10^{-7} mol l^{-1}) and mitochondrial KATP 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).*

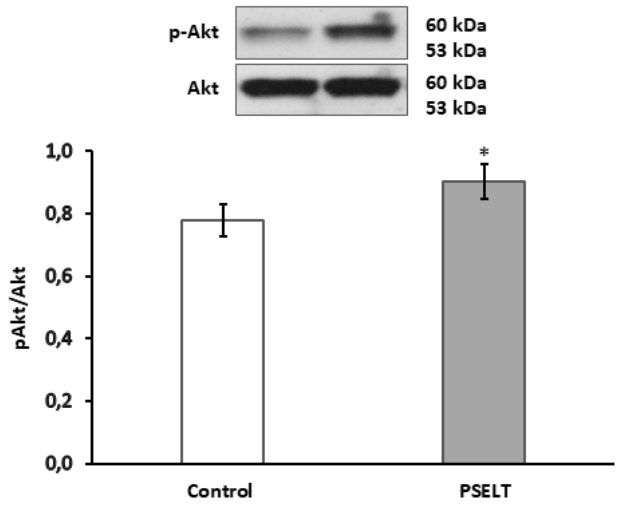

Figure 17. *p-Akt (Ser473) expression in goldfish cardiac extracts perfused with saline (control) or PSELT (10^{-10} mol l^{-1}). Values (phosphorylated versus non-phosphorylated Akt) are means± s.e.m. of three experiments for each condition. Significance of difference (unpaired t-test): *p<0.05.*

3.2.5. Cardiac SELENOT expression under hypoxia

Immunofluorescence studies performed by Prof. Y. Anouar at the University of Rouen, 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 (Figure 18). In addition, hypoxia provoked an increase in 3-NT, a marker of nitrosative stress, which was reversed by administration of PSELT (Figure 18).

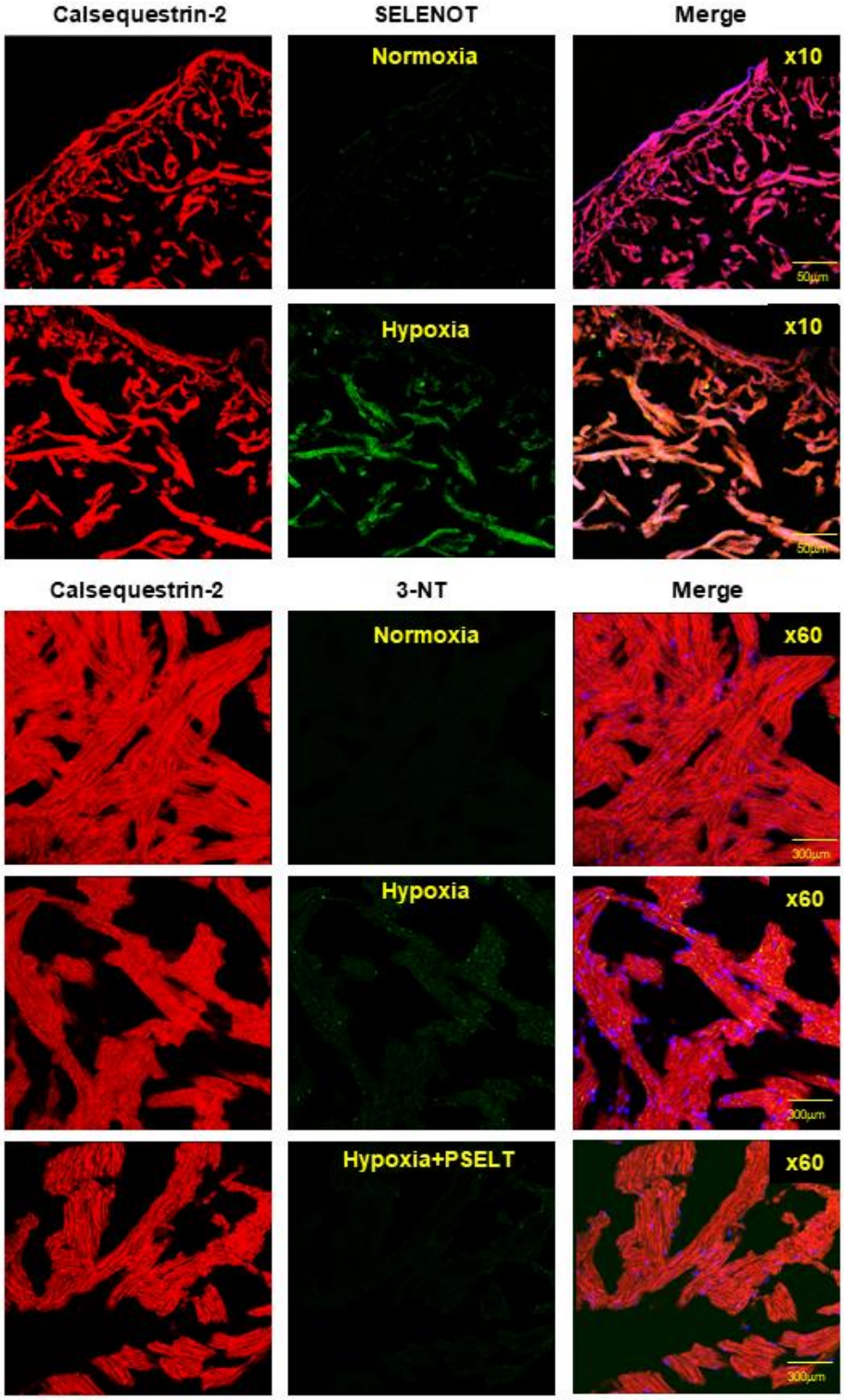

Figure 18. *SELENOT expression in the goldfish heart under normoxia and hypoxia. Top: representative images of SELENOT and calsequestrin-2 immunoreactivity under normoxia and hypoxia. Bottom: representative images of 3-NT and calsequestrin-2 immunoreactivity under normoxia, hypoxia and hypoxia in the presence of PSELT (10^{-10} mol l^{-1})*

3.2.6. 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}) an increase of SV and CO was observed (Figure 19).

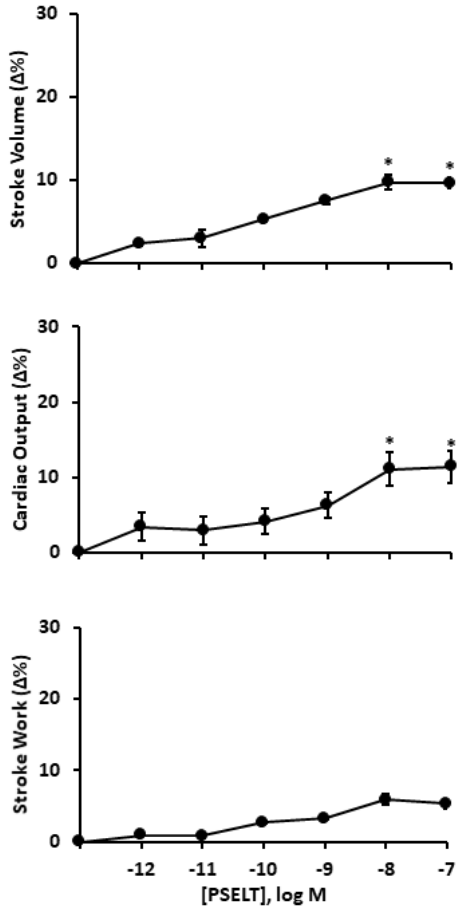

Figure 19. *Cumulative concentration–response curves for the effect of PSELT on stroke volume, cardiac output and stroke work in the isolated working goldfish heart perfused under acute hypoxia. Percentage change was evaluated as the mean ± s.e.m. of five experiments and is plotted against log PSELT concentration (mol l^{-1}). Significance of difference from control values (repeated measures ANOVA followed by Dunnett's post hoc test): *p<0.05*

3.3. 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 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.

3.3.1. 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. (2018) showed for the first time that SELENOT is highly expressed in embryos, decreases in new-borns 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., 2018). To evaluate whether SELENOT

expression in the adult heart is a trait peculiar to the goldfish, protein expression was also analyzed in cardiac extracts from other adult fish species. Western blot and densitometric analyses revealed that the protein is similarly present in cardiac homogenates from common carp (*C. carpio*), European eel (*A. anguilla*), zebrafish (*D. rerio*) and brown trout (*S. trutta*). 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 & 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., 2018), 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 foetal 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 foetal-like profile of the adult fish heart deserves further attention.

3.3.2. 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 SV, CO and SW, the effect being significant starting from 10^{-12} mol l^{-1}, and with the maximum increase (SV: 25.75±5.7%; CO: 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., 2018). 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 and colleagues (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 (Imbrogno, et al., 2014). 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; Vornanen , 1997; Vornanen, 1999; 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. Preliminary unpublished data (L. Boukhzar and Y. Anouar, manuscript in preparation) suggest that in neuroblastoma cells, 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; Gesser, 1996; Vornanen , 1997; 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 & 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 Thapsigargin, 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+} signalling and homeostasis in mammalian cells (Grumolato, et al., 2008; Pitts & 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 & 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., 2018). 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; Mazza, et al., 2013; Mazza, et al., 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.

3.4. 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, 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, this required the presence of an intact redox motif. Another similarity with mammals 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; 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.

CHAPTER 4.

MS-based proteomic analysis of cardiac response to hypoxia in the goldfish (Carassius auratus)

4.1. INTRODUCTION

Tissue energy unbalance, associated to a limited aerobic ATP production, is the main consequence of hypoxia exposure. To cope this metabolic alteration, an organism can adopt two strategies: to decrease energy consumption or to enhance anaerobic energy production (i.e. glycolysis). Since the latter has a lower yield of ATP than mitochondrial oxidative phosphorylation, to fully compensate for aerobic ATP production, anaerobic glycolytic flux must proceed at a rate of about 10 times oxidative phosphorylation. In addition, under O_2 limiting conditions, the loss of mitochondria capacity in producing ATP *via* oxidative phosphorylation increases the reduction of pyruvate to lactic acid by lactate dehydrogenase (LDH). The production of lactic acid is crucial for the oxidation of NADH back to NAD+ but, at the same time, imposes an intracellular acidic load which, in turn, can allow an allosteric proton inhibition of glycolytic regulatory enzymes (i.e. 6-phosphofructokinase). In the vertebrate heart, the hypoxia-related limited ATP production or increased intracellular acidification impair cardiac function with a consequent incapacity to maintain contractility (Rodnick & Gesser, 2017). However, some hypoxia tolerant species, such as the goldfish (*C. auratus*) and the epaulet shark (*Hemiscyllium ocellatum*), show the extraordinary capacity to increase cardiac contractility in response to low oxygen (Imbrogno, et al., 2014; Speers-Roesch, et al., 2012). Moreover, in the epaulet shark, the increased cardiac power output is associated with a low accumulation of cardiac lactate (Speers-Roesch, et al., 2012), which can reflect higher O_2 delivery to the heart during hypoxia exposure. Of note, in the goldfish, Regan and co-workers (2017) have recently shown that hypoxia exposure results in a transient increase in lactate, which subsequently (within few hours) recovers to values similar to the normoxic control (Regan, et al., 2017). This suggests the activation of specific mechanisms that, in this teleost contribute in reducing or avoiding lactate accumulation. It

is known that the goldfish is able to convert waste lactate in the harmless end product, ethanol, which in turn is easily excreted through the gills (Shoubridge & Hochachka, 1980). The production of ethanol exclusively occurs in the muscle, which possesses the biochemical machinery to process not only the lactate generated within the muscle itself, but also that produced by other tissues (brain, liver and heart) and delivered to the muscle *via* the blood flow (Fagernes, et al., 2017). However, this efficient mechanism does not fully justify the increase of cardiac performance observed in the isolated and perfused goldfish heart in response to hypoxia. Based on these premises, we evaluated the possibility that the exceptional hypoxia tolerance of the goldfish heart may be achieved through the activation of specific metabolic pathways which allow a reutilization of the first product of the anaerobic glycolysis (i.e. pyruvate), thus preventing lactate accumulation. To test our hypothesis, in collaboration with Prof. A. Napoli from the Department of Chemistry and Chemistry Technologies (CTC) at University of Calabria, we designed a classical mass spectrometry based proteomic study to identify, in the goldfish, cardiac proteins that may be associated with maintaining heart function under hypoxia. Particularly, we examined the cardiac proteomes, and evaluated the pyruvate concentration and the lactate dehydrogenase enzymatic activity in both normoxic and hypoxic goldfish heart.

4.2. RESULTS

Published in: *Scientific Reports (2019), 9,18953.* *Imbrogno, S.; Aiello, D.; Filice, M.;* ***Leo, S.****; Mazza, R.; Cerra, M.C.; MS-based proteomic analysis of cardiac response to hypoxia in the goldfish (Carassius auratus).*

To explore the possible functional roles (e.g., enzyme or biomarker) of the candidate proteins involved in goldfish cardiac adaptation to hypoxia, we established the experimental strategy reported in Figure 20. The strategy has two steps: MALDI MS, SDS MALDI MS/MS analysis and enzymatic assay on cardiac homogenates from goldfish hearts perfused under normoxic and hypoxic conditions. Enzymatic assay, i.e. pyruvate and lactate dehydrogenase assay, were used as orthogonal classic approach to validate mass spectrometry and bioinformatics output on cardiac sub proteome. Mass spectrometry and bioinformatic analyses were performed at the Department of CTC (Unical).

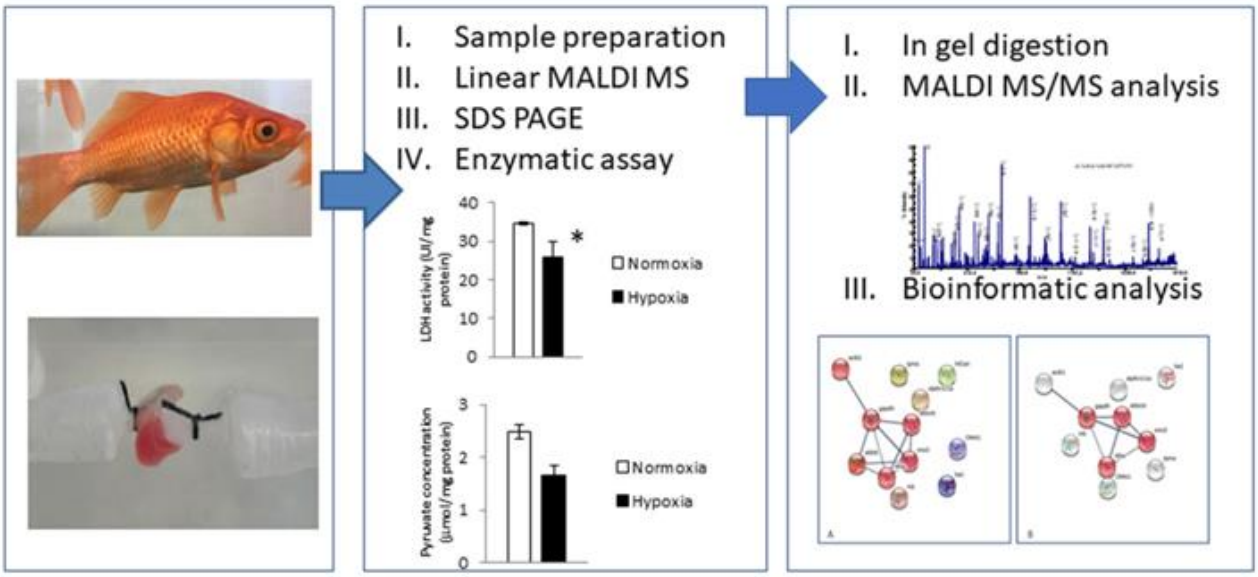

Figure 20. *Experimental strategy*

4.2.1. Protein extraction and identification

Few proteomic studies have been stated on goldfish (Tokumoto et al., 2000) using two-dimensional (2D) gel electrophoresis followed by in-gel digestion and mass spectrometric analysis (Reddy, et al., 2013; Gomes, et al., 2016). Literature data revealed the tri-modality feature for zebrafish (*Danio rerio*) heart proteome, with the majority of proteins centered around pIs 5.5 and 9 and fewer proteins around neutral pI (Zhang, et al., 2010). Furthermore, proteomic data on *Gillichthys mirabilis* cardiac tissues revealed 37 protein, involved in energy metabolism, mitochondrial regulation, iron homeostasis, cytoprotection against hypoxia, and cytoskeletal organization, with the majority of proteins centered around pIs 5.5–6.5 (Jayasundara, et al., 2015). The reliable detection of species-specific proteins and peptides, unique in mass and amino acid sequence, depends on proper protein solubilization, digestion, and sensitive MS analysis (Aiello, et al., 2016; Aiello, et al., 2018). Therefore, two different aqueous media were tested for the ability to extract proteins from the goldfish cardiac tissues: phosphate buffer (pH 7.5) and ammonium bicarbonate solution (50mM, pH 8). Proteins from goldfish cardiac tissues extracted with the two media were compared on 1-DE. A simple band pattern was obtained from both extracts with a low number of protein bands in each extract. The observed selective protein patterns generated by each extraction solutions emphasize the necessity of careful selection of the medium. Band patterns did not reveal significant differences among the extraction, suggesting a tissue specific sub-proteome denoted by chemically homogeneous protein families (Napoli, et al., 2006). The rapid and significant upgrading of sensitivity, throughput and mass accuracy of modern mass spectrometers drastically improved gel-free proteomic approaches. It is known that the accuracy of gel electrophoresis is frequently too low to be useful for mass measurement of intact proteins. MALDI MS has successfully been used for the direct analysis of peptides

and proteins in biological tissues (Rubakhin, et al., 2000), and the MS profiling has been applied to identify condition specific protein patterns in fish tissues and organs (Provan, et al., 2006). The molecular distribution acquired by MS provide important data to highlight physiological responses associated to environmental changes (Provan, et al., 2006), and the MS-based chemical component profiling represents a powerful tool to obtain precious information. The direct MS analysis of tissue extracts has been found to be a useful and robust approach to identify features (Duncan, et al., 2016) (m/z value and their relative peak heights/areas) within spectra that differentiate between distinct groups (e.g., spectra derived from normoxic vs hypoxic conditions). Moreover, the direct desorption of proteins from crude extracts shows the advantage of minimizing sample loss during its workup. The selection of an appropriate matrix/sample preparation method is necessary to obtain high-quality mass spectra from nanomolar (50–100 nM) protein samples. The singly protonated molecule $[MH]^+$ is predominant ion species in the MALDI mass spectrum, however multiply charged ions (doubly and triply charged $[M+2H]^{2+,}$ $[M+3H]^{3+}$) are progressively evident as protein molecular mass increases. It is known that matrices with higher ionization energy, such as CHCA, generate multiply charged protein ions. For the direct MS analysis of crude extracts presented here, CHCA [5 mg/mL; sample/matrix: 1/2 (v/v)] was chosen in order to obtain the highest analyte sensitivity, favoring the formation of doubly and triply charged protein ions. The formation of multiply charged ions in linear MALDI experiments represents a valuable advantage for the accurate protein mass determination. Although chemical composition and molecular mass affect the ionization efficiency, and consequently the relative peak intensities, the magnitude of the MS signals reflects the relative amounts of mixture components. The relative low number of protein ion signals which can be detected in spectra agrees the experimental protocol adopted for tissue

extraction. Representative linear MALDI MS spectra are reported in Figure 21A,B. Figure 21B displays linear MALDI mass spectrum of hypoxic cardiac tissue extract (4 ÷ 60 kDa). The presence of single and multicharged protein ions allowed the identification of twelve proteins with 0.1% ÷ 0.6% accuracy (Table 4, column 1). In order to compare protein profiles of hypoxic and normoxic cardiac tissue extracts, the obtained MALDI-MS spectra were exported as ASCII spectra (S/N = 5; Data Explore 4.1). The spectra were aligned by setting centroid peak shift to 0.1% of the mass over charge (m/z) value, and the average molecular mass (using +1, +2 and +3 ion signals) of each mixture component was determined. The protein hypoxic and normoxic profiles exhibited such a few differences, the most relevant was found in the mass range between 38.5 kDa and 40 kDa. The protein signal of m/z 39163 ± 153 Da detected in hypoxic cardiac tissue extracts was absent in normoxic tissue extracts, which instead exhibited a protein signal at 39668±83 Da. The difficult matching of the experimentally determined and theoretical molecular weights of hypothetical proteins, as predicted through genome annotations, could represent a limitation. Several factors may cause this discrepancy, and they include polymorphism in the protein sequence between the species, incorrect genome annotation, redundancy or errors in the amino acid sequences, and possible post-translational modifications. The top-down approach utilizing MS/MS experiments on single and double charged ions signals did not allowed the identification of proteins. The concentration of proteins present in low amounts in the extracts, limits the applicability of top-down experiments even though the mixtures obtained from tissue extractions showed a low complexity. Despite the above technical restrictions in the full identification of the cardiac proteins (which was beyond the scope of this work), the results demonstrate that MALDI-MS can suitably be used in the direct analysis of tissue extracts, allowing the detection of the specific protein pattern associated to the physiological

conditions of each individual. Protein gel image analysis revealed 7 distinct protein bands in both groups (i.e. normoxic and hypoxic goldfish cardiac tissues). Tryptic peptides obtained by in gel digestion were loaded on to MALDI plate and analyzed in MS and MS/MS mode. The identification of protein parent was performed using Protein Pilot Paragon Method and the results of the database search are listed in Table 4. MS/MS data were processed using a mass tolerance of 0.2 Da and 10 ppm for the fragment ions and precursor, respectively. The phosphorylation of serine and threonine was included in the variable modifications and all spectra were manually evaluated. From the two set of protein bands, we were able to identify 12 proteins using mass spectrometry (Table 4). The detection of well-known hypoxia-induced proteins (up-regulated) such as glyceraldehyde-3-phosphate dehydrogenase (GAPDH), L lactate dehydrogenase (LDHA), phosphoglycerate Hypoxia-inducible factor 1-alpha inhibitor (HIF1N), Tropomyosin alpha-1 chain (TPM1) and beta-enolase (ENOB) was indicative of the robustness of our experimental approach.

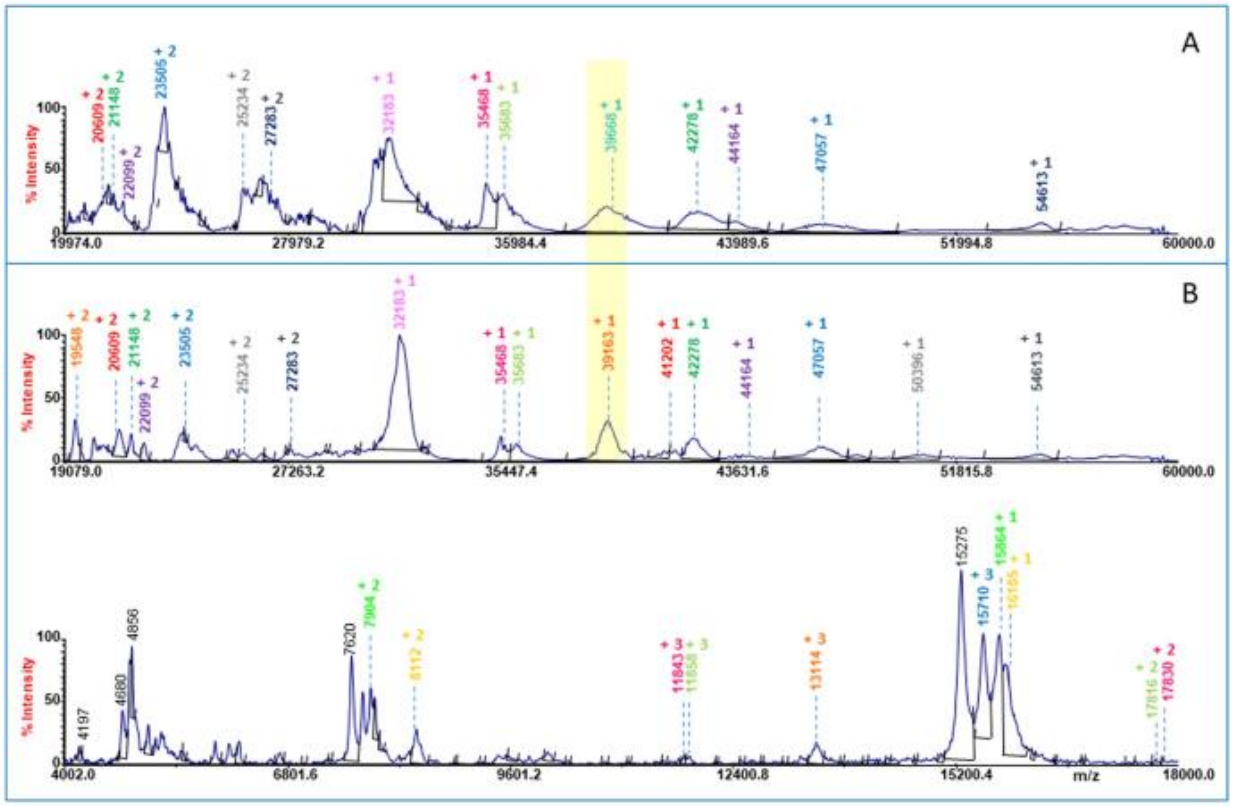

Figure 21. *Linear MALDI MS of normoxic (**A**) and hypoxic (**B**) cardiac tissue extract*

	MW MS	MW[a]	PI[b]	Protein ID	*Name*	Normoxia	Hypoxia
1	44164 ± 45	44315	6.93	VTC1A_DANRE	*V-type proton ATPase subunit C 1-A*	+	+
2	47057 ± 62	47287	6.61	ENOB_SALSA	*Beta-enolase*	+	+
3	39163 ± 153	39288	8.48	ALDOB_ DANRE	*Fructose-bisphosphate aldolase B*	–	+
4	39668 ± 83	39449	6.41	ALDO_CARAU	*Fructose-bisphosphate aldolase C*	+	–
5	42278 ± 22	41971	5.23	ACTS_CARAU	*Actin, alpha skeletal muscle*	+	+
6	32183 ± 49	32723	4.70	TPM1_DANRE	*Tropomyosin alpha-1 chain*	+	+
7	35468 ± 224	35784	8.19	G3P_DANRE	*Glyceraldehyde-3-phosphate dehydrogenase*	+	+
8	35683 ± 147	36334	6.70	LDHA_FUNHE	*L-lactate dehydrogenase B-A chain*	+	+
9	15864 ± 82	15851	7.89	B3CJI6_CARAU	*Myoglobin*	+	+
10	16185 ± 52	16210	7.85	HBB_CARAU	*Hemoglobin subunit beta*	+	+
11	54613 ± 69	54050	9.69	OMA1_DANRE	*Metalloendopeptidase OMA1, mitochondrial*	+	+
12	41202 ± 21	39992	5.66	HIF1N_DANRE	Hypoxia-inducible factor 1-alpha inhibitor	–	+
13	50396 ± 140	—	—	Unknown		–	+

Table 4. *Protein identified by MALDI MS experiments and after in gel digestion- MALDI MS and MS/MS*

4.2.2. Bioinformatic analysis

Gene ontologies and pathways analysis by PANTHER revealed the presence of 4 different protein classes. The most prominent classes were cytoskeleton proteins (25%), hydrolases (25%), lyases (12,5%), oxidoreductases (25%) and transporters (12,5%). The identified proteins were listed using the gene name and the ZFIN ID for the analogous reference species *Danio rerio.* The final dataset was subsequently investigated by gene ontology, network analyses. The metabolic pathways were investigated by DAVID tools. The top score pathways included proteins involved in (a) glycolysis/gluconeogenesis, (b) biosynthesis of antibiotics, (c) biosynthesis of amino acids, (d) carbon metabolism, and (e) metabolic pathways. A large group of proteins (45%) was found to be involved in glycolysis/gluconeogenesis pathway. Proteins associated with this pathway were *aldocb, eno3, gapdh, ldha*. The study of functional domains by InterPro Motifs performed by the DAVID software discovered that the top protein motifs corresponded to globin and globin like structural domains. Globins are heme-containing proteins ubiquitously expressed in vertebrates, including fish, in which they play a broad range of biological functions, directly or indirectly related to the tight control of oxygen levels and its toxic products (Fago, 1985). The biological processes associated with the identified proteins of goldfish were (a) glycolytic process, (b) response to hypoxia, (c) glucose metabolic process. Evaluation of the biological functions by STRING revealed 8 top biological processes, including (a) carbohydrate metabolic process, (b) response to hypoxia, (c) pyruvate metabolic process, (d) glycolytic process. The comparison between normoxic and hypoxic goldfish cardiac proteome evidenced the “carbohydrate metabolic process” and “response to hypoxia” as the major biological processes. These results are in accordance with the high number of enzymes (5) identified. All proteins identified for both hypoxic and normoxic proteome

were subjected to STRING database (https://string-db.org/). Only the interactions tagged as "high confidence" (0.7) in STRING database, have been considered to minimize false positives as well as false negatives. For hypoxia, the network is composed by 12 nodes (proteins) and 10 edges (interactions); expected number of edges 0 and PPI enrichment p-value: 9.52e-13 (Figure 22). To create this network, a value of 3 for the MCL clustering coefficient was chosen.

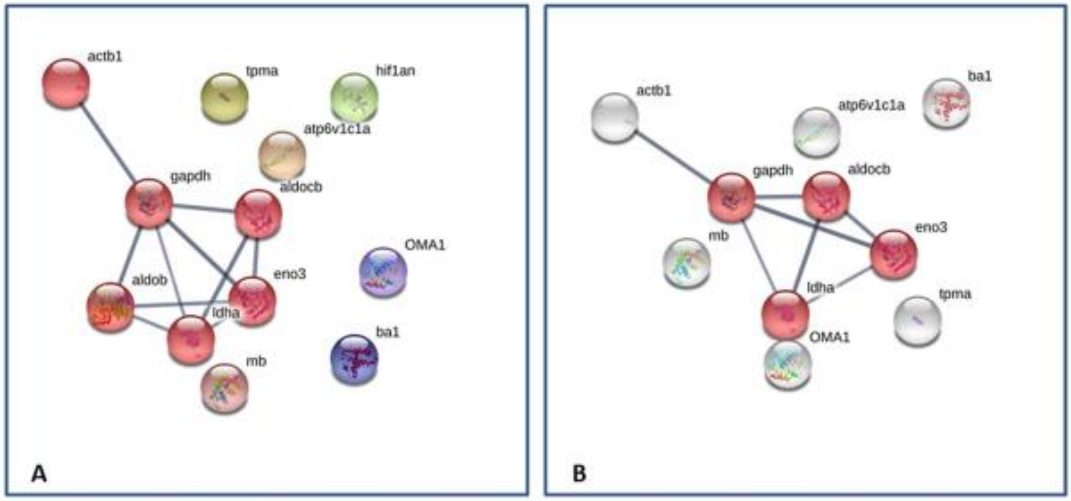

Figure 22. *Protein-Protein interaction network by STRING software (panel A: Hypoxia; panel B: Normoxia).*

4.2.3. Enzymatic assay to validate mass spectrometry and bioinformatic results on cardiac sub proteome

To validate mass spectrometry and bioinformatic results on cardiac sub proteome, we measured pyruvate concentration and LDH activity in homogenates of goldfish hearts perfused either under normoxic or hypoxic conditions. Results showed that, with respect to the normoxic counterpart, lower levels of LDH activity were measured in homogenates of hearts exposed to hypoxia (hypoxia: 25.9 ± 4 UI/mg protein; normoxia: 34.56 ± 0.24 UI/mg protein; Figure 23). This hypoxia-dependent reduction of LDH activity resulted associated to a slight, although not significant, reduction in pyruvate levels (hypoxia: 1.65 ± 0.21

µmol/mg protein; normoxia: 2.49±0.84 µmol/mg protein; Figure 23). This suggests that under hypoxia, the conversion of pyruvate to lactate is less effective; however, it is not associated with pyruvate accumulation.

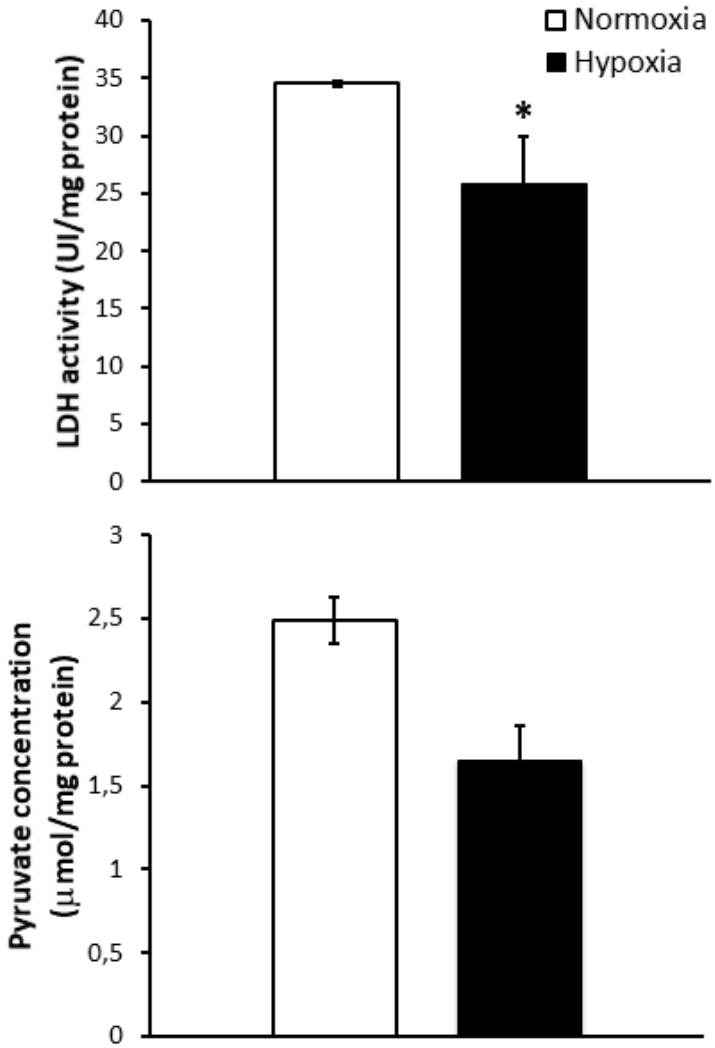

Figure 23. *LDH activity (upper panel) and Pyruvate concentration (lower panel) in goldfish (C. auratus) hearts perfused either under normoxic or hypoxic conditions. Values are the mean ± s.e.m. of three individual experiments for each condition. Statistics are assessed by unpaired t-test (*p <0.05).*

4.3 DISCUSSION

In the study here reported, mass spectrometry followed by bioinformatics analysis and enzymatic assays has been used to study *ex vivo* proteome of normoxic and hypoxic goldfish cardiac tissues. MS-based proteomics is a rapid and sensitive tool for the identification (Jahouh, et al., 2010) and quantitation (Di Donna, et al., 2015; Napoli, et al., 2010) of proteins in specific functional contexts. The experimental approach here reported was designed with the purpose to identify a set of the most stable known hypoxia-regulated proteins. This functionally oriented strategy, focused on the search for acid hydro-soluble proteins, was driven by the hypothesis that the exceptional hypoxia tolerance of the goldfish heart may be accomplished through the activation of alternative mechanisms. MS/MS analysis of goldfish hearts sub-proteome has allowed to identify the hypoxia-regulated protein composition under both normoxia and hypoxia (Table 4). Among the well-known hypoxia-induced proteins, we identified a novel hypoxia-regulated protein fructose-bisphosphate aldolase B (ALDOB_ DANRE), which was found to be expressed in *ex vivo* hypoxic cardiac samples. In particular, MS/MS assignment of 40 kDa protein spots from hypoxic and normoxic conditions corresponds to fructose-bisphosphate aldolase B (ALDOB_ DANRE) and fructose-bisphosphate aldolase C (ALDOC_ CARAU), respectively. Sequence information was obtained with tandem MS of 18 peptides. These peptides fully match the aldoc (ALDOC_ CARAU) sequence and cover 51% of the 363-amino acid protein. Similarly, 18 peptides match the aldob (ALDOB_ DANRE) sequence and cover 68% of the 364-amino acid protein. Aldob and aldoc are isoforms of fructose-bisphosphate aldolase sharing 73.901% sequence identity. The relatively large number of amino acid differences between aldoc and aldob (e.g. aldoc has 95 mutations compared with the aldob enzyme) make straightforward the identification of the two enzymes. For example, the point mutations of

^{315}K → R, ^{317}Q → V, ^{318}A → K, ^{319}A → E and ^{322}K → E (aldob *vs* aldoc) were univocally assigned by a single MS/MS experiment. The fragmentation pattern of the sequence ^{305}ALQASALSAWRGVKENEK^{322} ($[M - H]^+$ of m/z 1958.06, Δppm = 5.9; Figure 24A) shows the complete b-type ion series allowing the identification of ALDOC_CARAU in normoxic condition. Similarly, the detection of the ion of m/z 1791.99 (Δppm = 5.7; Figure 24B) from 40 kDa digested obtained in hypoxic condition allowed the identification of aldob. The sequence ^{244}YTPLEVAMATVTALRR^{259}, deduced from the fragmentation pattern of m/z 1791.99, featured the point mutations at the positions 244 (F → Y), 245 (S → T), 246 (N → P), 247 (L → Q) and 248 (I → V) (aldoc *vs* aldob). Therefore, the aldob (ALDOB_DANRE) was assigned as the protein source of the peptide sequence ^{244}YTPLEVAMATVTALRR^{259} on the basis of the sequence congruency with the SwissProt database. In mammalian tissues, three aldolase isoforms have been identified: aldolase A, the classical muscle enzyme; aldolase B, a form predominantly expressed in the liver; aldolase C, whose expression has been observed in the brain. Unlike aldolase A, which appears to be more effective in participating to glycolysis and aldolase C which, although possessing intermediate properties, seems to be utilized mainly in the cleavage of fructose-1,6-bisphosphate, aldolase B has evolved to have a role in gluconeogenesis (Penhoet, et al., 1969; Penhoet & Rutter, 1971; Eagles & Iqbal, 1973). Metabolic state and substrates availability have been proposed to modulate the expression of aldolase isoforms, and thus of glucose synthesis or degradation (Droppelmann, et al., 2015). The protein network highlighted in Figure 22A showed the main set composed by 5 nodes (*aldob, aldocb, eno3, gapdh, ldha*) which results in accordance with the top canonical pathway previously obtained by DAVID. These nodes are referred to enzymes catalyzing reversible steps of the glycolysis/gluconeogenesis network. No interaction was observed for the HIF-1α inhibitor (FIH-1). HIF-1α is the major

transcriptional regulator of the cellular hypoxia response. Its binding to DNA as an α/β heterodimer is regulated by oxygen dependent prolyl (PHD) and asparaginyl (FIH-1) hydroxylases. PHD is involved in the stability of HIF–α sub-unit, while FIH-1 regulates its transcriptional activity. Unlike PHD enzyme, whose activity is completely suppressed by hypoxia, FIH-1 remains active even at relatively low oxygen levels. The ability of the hypoxic cyprinid fish to maintain a normal (*C. carassius*) (Stecyk, et al., 2004a), or even enhanced (*C. auratus*) (Imbrogno, et al., 2014), cardiac performance has been related to the capacity to cope with acidosis associated with anaerobic end-products accumulation (e.g. lactate) (Gattuso, et al., 2018). This may occur either through a metabolic removal of lactate and/or to the recruitment of pyruvate in alternative metabolic pathways (e.g. gluconeogenesis). Several evidences suggest that the goldfish, as well as its relative, the crucian carp, is able to reduce lactate to ethanol, an acid–base neutral molecule, easily excreted by the gills (Shoubridge & Hochachka, 1980). The production of ethanol exclusively occurs in the swimming muscle, which possesses the biochemical machinery to process not only the lactate generated within the muscle itself, but also that produced by other tissues such as brain, liver and heart and delivered to the muscle *via* the blood flow (Dhillon, et al., 2018). In the whole organism, this mechanism efficiently allows the conversion of waste lactate in a harmless anaerobic end product. However, it takes time, depending on the physiological circulatory interactions between organs and tissues. Moreover, it does not fully justify the increase of cardiac performance observed in the isolated and perfused goldfish heart in response to hypoxia (Imbrogno, et al., 2014). Indeed, according to mammalian paradigms, in an isolated organ (i.e. a "closed system"), anaerobic lactate and H^+ accumulation should lead to intracellular acidosis, impairment of myocardial contractility/relaxation and hence a dramatic decrease of the heart ability to pump blood

(Orchard & Kentish, 1990; Orchard & Cingolani, 1994). In this context, and in agreement with the bioinformatics evidence of the interaction between lactate dehydrogenase A (ldha) and fructose-bisphosphate aldolase B (aldob), our biochemical assays allows to propose also a role of gluconeogenesis in the mechanisms involved in the response of the goldfish heart to hypoxia (Figure 25). The hypoxia-induced activation of glucose re-synthesis, allowing a continuous recycle of pyruvate, contributes to prevent the production of deleterious wastes, thus assuring the maintenance of cardiac pumping ability.

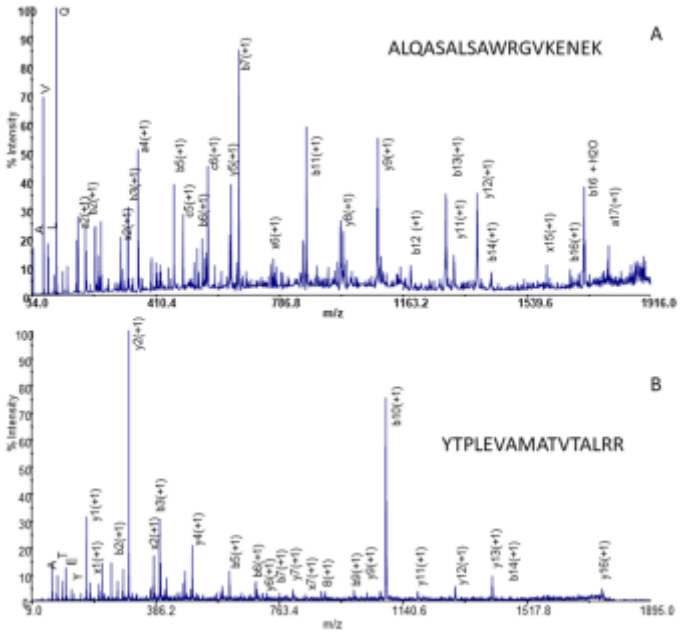

Figure 24. *MALDI MS/MS spectrum of (**A**) m/z 1958.06 ([M - H]* $^{+}$*) and (**B**) of m/z 1791.99 ([M - H]*$^{+}$*).*

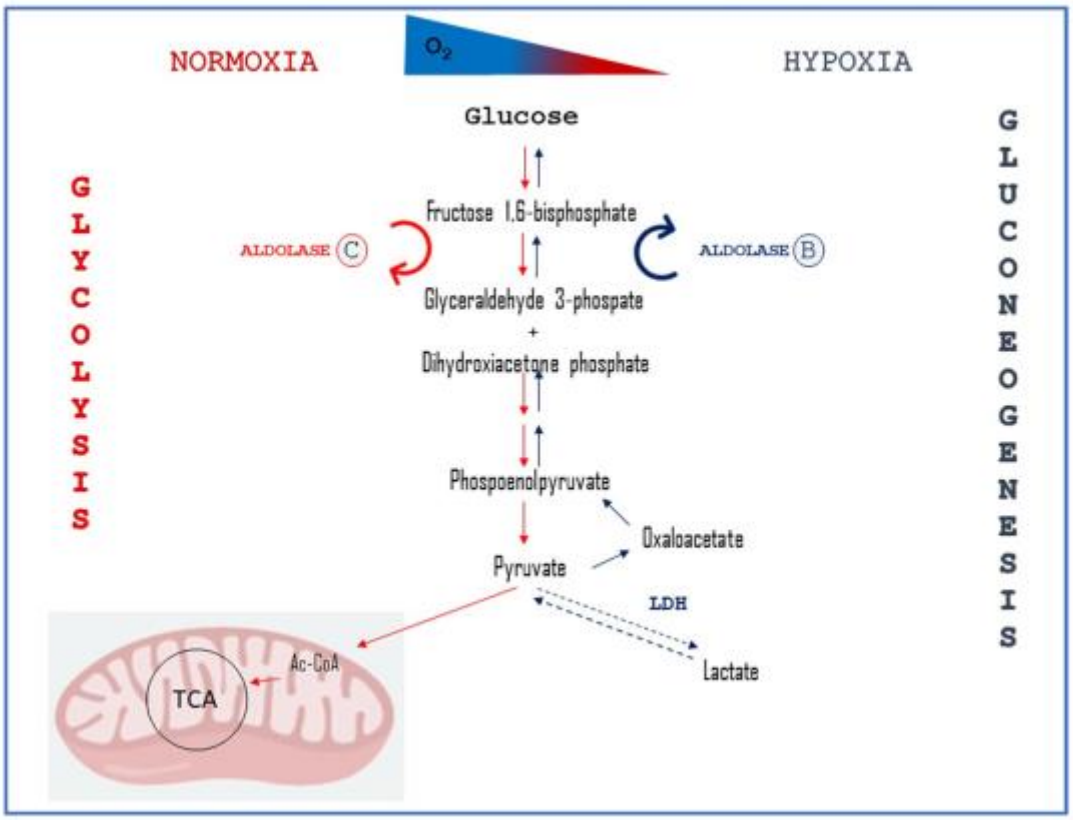

Figure 25. *Model for alternative routes of pyruvate metabolism in the goldfish (C. auratus) heart. Pyruvate from glycolysis is either converted to acetyl-CoA (Ac-CoA) (under normoxia) or to lactate by the lactate dehydrogenase (LDH) (under hypoxia). As suggested by our analysis, under low oxygen levels, LDH activity is reduced (dashed arrows), and pyruvate may be converted in oxaloacetate and re-used in gluconeogenesis.*

4.4. CONCLUSIONS

In this work, we perform a selective extraction of goldfish heart proteins followed by tandem mass spectrometric analysis. The observed protein composition proves that the adopted experimental condition is an effective alternative to the classic extraction methods to look at the most stable proteins. This approach led the identification of 12 most stable hypoxia regulated proteins. It represents the first experimental report of *in vitro* normoxic and hypoxic goldfish cardiac tissues, proving that aldolase B may be a novel hypoxic marker. The findings agree with the hypothesis that under conditions of reduced oxygen, a modulation of the aldolase enzyme isoforms allows the goldfish heart to recycle the first product of the anaerobic glycolysis, namely pyruvate. This may minimize deleterious waste accumulation, allowing, at the same time, to mitigate the negative consequences of a low hypoxia-dependent ATP production. Our data suggest that, in the goldfish heart, a highly coordinated regulatory system may fine-tune glycolytic vs gluconeogenic flux in relation to oxygen availability, thus contributing to hypoxia tolerance.

GENERAL CONCLUSIONS

Results obtained during my PhD research activity gave a contribute in revealing aspects of the mechanisms that in the goldfish *Carassius auratus* modulate cardiac performance under hypoxia.

In particular, the crucial role exerted by the β3-AR and the NOS/NO system as upstream and downstream, respectively, mechanisms activated to sustain hypoxia tolerance, the protective role exerted by new emerging antioxidant proteins on the hypoxic myocardium, as well as some mechanisms of metabolic adaptation to sustain cardiac contractility in response to hypoxic stress, have been highlighted.

Overall, the results suggest a highly coordinated regulatory mechanisms which in the goldfish heart control organ, tissue, molecular and metabolic adaptation to hypoxia.

Although much remains to be clarified, we hope that these results may represent a rational basis for better investigating the complex molecular networks involved in the cardiac response to hypoxia in fish, also contributing, in an evolutionary and translational perspective, to better decipher the mechanisms that in the mammalian heart may improve resistance to hypoxia, such as in conditions of myocardial ischemia.

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