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"Cardiac plasticity in fish: the influence of humoral and environmental factors"

PhD student

Dott. Mariacristina Filice

Supervisor

Prof. Sandra Imbrogno

Coordinator

Prof. Maria Carmela Cerra

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SOMMARIO

Il rimodellamento cardiaco è un fenomeno complesso che permette un'adeguata attività d'organo in risposta a cambiamenti fisiologici e patologici e coinvolge modificazioni a livello tissutale, cellulare e molecolare, secondo meccanismi ancora poco noti. Il cuore dei vertebrati mostra una notevole capacità di adattarsi funzionalmente alle richieste emodinamiche dell'organismo. Questa plasticità è esemplificata dalla modulazione "beat-to-beat" della contrattilità in risposta a cambiamenti del carico pressorio e volumetrico (risposta di Starling) e dal riarrangiamento morfo-funzionale adattativo. I maggiori determinanti della risposta plastica del cuore sono gli stimoli fisici come lo stress meccanico e lo stiramento dei miociti, o chimici, come ad esempio quelli esercitati da cardiomodulatori, inclusi i Peptidi Natriuretici (NPs), l'endotelina-1 (ET-1), l'Ossido Nitrico (NO) e l'Angiotensina II (AngII). Queste molecole attivano specifici circuiti endocrini/paracrini/autocrini responsabili del controllo omeostatico della funzione e della crescita cardiaca. Nei mammiferi, il cuore adulto è considerato un organo terminalmente differenziato nel quale la crescita e il rimodellamento avvengono per ipertrofia. Questo paradigma è stato recentemente confutato da evidenze che mostrano come, durante la normale crescita cardiaca, i miocardiociti adulti proliferano e muoiono. L'identificazione di stimoli e meccanismi che, attivando programmi genetici e/o metabolici silenti, possano permettere ai cardiomiociti di mammifero adulto di proliferare, è al momento tra gli scopi più importanti della ricerca cardiovascolare moderna. A tal riguardo un possibile approccio è quello di identificare esempi di plasticità cardiaca fra i vertebrati.

Negli ultimi anni il cuore dei pesci ha rappresentato un importante strumento di ricerca per analizzare aspetti molecolari, cellulari e tissutali della plasticità cardiaca. Nei pesci, il cuore adulto conserva la capacità di crescita iperplastica in risposta a cambiamenti delle condizioni ambientali, all'esercizio fisico e alla maturità sessuale. Esempio estremo di questa plasticità è rappresentato dalla completa rigenerazione del cuore di zebrafish in seguito a rimozione chirurgica di una porzione del ventricolo. In una prospettiva traslazionale, i risultati di studi effettuati nei pesci potrebbero rappresentare uno strumento importante per decifrare i determinanti e i meccanismi coinvolti nel rimodellamento morfo-funzionale del cuore dei mammiferi.

Partendo da queste premesse, questo progetto di tesi ha voluto valutare se e in che misura, fattori umorali e ambientali influenzano la plasticità cardiaca dei pesci.
In particolare, in questa tesi di dottorato sono riportati e discussi i risultati ottenuti da studi relativi al controllo umorale del rimodellamento morfo-funzionale cardiaco mediato dall'esposizione cronica all'AngII nel cuore dell'anguilla europea *Anguilla anguilla* e dello zebrafish *Danio rerio*, e i meccanismi fisiologici e molecolari attivati nel cuore della carpa, *Carassius carassius*, in risposta all'ipossia.
L'AngII, il prodotto bioattivo del Sistema Renina Angiotensina (RAS) è un potente ormone le cui azioni biologiche sono state ampiamente studiate nei mammiferi. A livello cardiaco, legandosi a recettori di tipo AT1 e AT2, l'AngII influenza positivamente la contrattilità miocardica e regola la crescita miocitaria. Un RAS omologo a quello dei mammiferi è presente anche nei pesci. In particolare, nei teleostei, l'AngII può indurre modificazioni cardiache a breve (modulazione della contrattilità) e lungo termine (rimodellamento morfo-funzionale). Nel 2013, Imbrogno e collaboratori hanno dimostrato che il cuore di anguille esposte per 4 settimane ad iniezioni intraperitoneali di AngII mostrava una migliore capacità di rispondere ad incrementi di post-carico. Quest'effetto era mediato dall'attivazione dei recettori AT2 e accompagnato da una differente espressione e localizzazione di proteine coinvolte nella regolazione della crescita e dell'apoptosi. Partendo da queste premesse, questo studio ha voluto meglio investigare le modificazioni strutturali e molecolari attivate nel cuore di anguille esposte per 8 settimane ad iniezioni intraperitoneali di AngII. Analisi morfologiche hanno evidenziato che i cuori di animali trattati mostravano un incremento della muscolarità ventricolare associato ad un aumento dello spessore del compatto e ad una riduzione degli spazi lacunari nello spugnoso. Queste modificazioni erano accompagnate da un'incrementata vascolarizzazione del compatto. Analisi di western blotting e immunofluorescenza hanno evidenziato un'aumentata espressione del recettore AT2 nei cuori di animali trattati, associata ad una diversa localizzazione: nei controlli AT2 localizza principalmente a livello miocitario mentre in seguito a trattamento, un segnale fluorescente si osserva a livello dell'endotelio endocardico. In relazione al ruolo cruciale svolto dallo NO nei meccanismi di regolazione del rimodellamento cardiaco nei pesci, lo studio ha voluto anche analizzare il cross-talk tra AngII e il sistema NO Sintasi (NOS)/NO. In condizioni basali, lo NO prodotto dalla NOS viene

immediatamente metabolizzato a nitriti o nitrati. Per questa ragione, il dosaggio dei nitriti può essere utilizzato per misurare i livelli di NO prodotti dalla NOS e di conseguenza la funzionalità stessa dell'enzima. Il dosaggio dei nitriti, effettuato su omogenati di cuori di animali trattati con AngII ha evidenziato una riduzione significativa della concentrazione di nitriti rispetto ai controlli, indice di una ridotta attività della NOS. Analisi di western blotting non hanno evidenziato variazioni significative nei livelli di espressione della forma fosforilata (e quindi attiva) della NOS. Tuttavia, il trattamento ha influenzato la localizzazione dell'enzima fosforilato. Infatti, mentre nei controlli peNOS localizza sia nei miociti che a livello dell'endotelio endocardico (EE), nei trattati si osserva una maggiore localizzazione a livello miocitario e solo un debole segnale a livello endocardico. Questo dato è accompagnato da una maggiore espressione, a livello dell'EE, di NOSTRIN, un modulatore negativo della NOS, responsabile della traslocazione dell'enzima dalla membrana plasmatica all'interno di vescicole citoplasmatiche. Analisi di western blotting hanno anche evidenziato una riduzione nei livelli di espressione della chinasi Akt e dello chaperone HSP90, due tra i principali modulatori positivi della NOS. Nel complesso questi risultati dimostrano che il cuore di anguilla cronicamente esposto all'azione dell'AngII va incontro ad un riarrangiamento strutturale e ad una modulazione dei livelli di NO e delle proteine che regolano l'attività della NOS.

Gli effetti cronici dell'AngII sono stati investigati anche nello zebrafish. Sebbene questo studio è ancora in corso, i primi risultati sperimentali dimostrano che anche il cuore di zebrafish va incontro ad un rimodellamento strutturale se esposto per 8 settimane all'azione dell'AngII. In particolare, i cuori trattati con AngII mostrano un incremento del compatto e una riduzione degli spazi lacunari, indicativi di un aumento della muscolarità ventricolare. Questo è associato ad un aumento del collagene e ad un significativo aumento del peso del cuore e dell'indice cardiosomatico (rapporto in percentuale tra il peso del cuore e il peso dell'animale). Analisi di western blotting hanno evidenziato inoltre un'aumentata espressione di entrambi i recettori AT1 e AT2 in omogenati cardiaci di animali trattati con AngII.

La seconda parte della tesi ha analizzato i meccanismi fisiologici e molecolari attivati nella carpa in risposta all'ipossia.

A differenza dei mammiferi, alcune specie di pesci, come ad esempio i ciprinidi, mostrano una notevole capacità di sopravvivere e restare attivi anche per lungo

tempo se esposti a condizioni di ipossia o anossia. Questa capacità è legata alla loro abilità di mantenere o aumentare la performance cardiaca in risposta ad una riduzione di ossigeno ambientale, favorendo così gli scambi tra i tessuti. Inoltre, questi pesci sono in grado di convertire il lattato (prodotto ultimo della glicolisi) in etanolo, che è poi eliminato attraverso le branchie. Una caratteristica dei pesci è la loro capacità di captare, attraverso le branchie, nitriti e nitrati dall'acqua in cui si trovano; in condizioni di ipossia, quando l'attività della NOS è compromessa, i nitriti possono rappresentare un'importante fonte di NO che, a sua volta, interviene nei processi di citoprotezione. Sulla base di queste osservazioni, in collaborazione con il Prof. Jensen (Università di Odense) e la Prof.ssa Fago (Università di Aarhus), è stato avviato uno studio volto a valutare il ruolo dei nitrati e della mioglobina come fonti alternative di nitriti, e quindi di NO, nella carpa esposta per un giorno ad ipossia profonda ($1<PO_2<3$ mmHg). Omogenati cardiaci e di muscolo rosso e bianco hanno evidenziato un aumento di nitriti e di altri metaboliti dello NO, quali Fe-nitrosile (FeNO) S-nitroso (SNO) e N-nitroso (NNO), sia nel cuore che nel muscolo rosso di carpe esposte ad ipossia. Questo incremento non era correlato a differenze nelle concentrazioni di mioglobina. Il dosaggio dell'attività nitrato reduttasica tissutale in omogenati di cuore, muscolo bianco e fegato, ha inoltre evidenziato che in condizioni di ipossia profonda, il muscolo e il fegato sono in grado di convertire il nitrato esterno in nitrito. Questa attività non è stata riscontrata nel cuore, dove risulta invece evidente una riduzione nelle concentrazioni di nitriti in risposta all'ipossia. Questi risultati supportano il ruolo dei nitriti come fonte alternativa per la generazione di NO durante l'ipossia; questo risulta maggiormente evidente in tessuti ricchi in mioglobina e mitocondri come il cuore, dove la conversione di nitriti a NO potrebbe rappresentare un importante meccanismo di cardioprotezione. Inoltre, in condizioni ipossiche, l'attività nitrato reduttasica riscontrata nel muscolo e nel fegato degli animali esposti ad ipossia potrebbe essa stessa supplementare le riserve intracellulari di nitriti contribuendo alla citoprotezione.

In conclusione, i risultati riportati in questa tesi suggeriscono che se esposto a influenze umorali o ambientali il cuore dei pesci va incontro ad un significativo rimodellamento molecolare, strutturale e fisiologico. Sebbene molto resti ancora da chiarire sui meccanismi molecolari attivati, questi risultati indicano chiaramente un ruolo chiave svolto dal sistema NOS/NO come principale coordinatore/integratore

di cascate molecolari che controllano le risposte cardiache adattative nei pesci. Ricerche future contribuiranno a meglio decifrare i complessi networks molecolari coinvolti nella plasticità cardiaca di questi vertebrati. In questo contesto, la scelta di modelli sperimentali appropriati, caratterizzati da una marcata adattabilità morfo-funzionale ad una varietà di stimoli ambientali (temperatura, pH, pressione parziale di ossigeno, etc.) potrà essere di grande aiuto.

PREFACE

Cardiac morpho-functional remodelling: the power of natural animal models

Cardiac remodelling may be defined as genome expression, molecular, cellular and interstitial changes that are manifested as changes in size, shape and function of the heart in response to physiological and/or pathological conditions (Cohn et al., 2000). In mammals, exercise, pregnancy, and postnatal growth promote physiologic growth of the heart, whereas neurohumoral activation, pressure overload (aortic stenosis, hypertension), inflammatory heart muscle diseases (myocarditis) and myocardial injury could be causes of pathologic hypertrophic growth. Likewise, protracted bed rest, mechanical unloading with a ventricular assist device and prolonged weightlessness during space travel, are causes of cardiac atrophy (Hill and Olson, 2008) (Fig. 1). Although the etiologies of these diseases are different, they share several pathways in terms of molecular, biochemical and mechanical events. Physiological cardiac hypertrophy can lead to an increase in heart size characterized by normal cardiac morphology with a normal and/or enhanced cardiac function (Ooi et al., 2014). By contrast, pathological hypertrophy is characterized by thickening of ventricular wall, decrease in the size of the cardiac chambers and reduced capacity of the heart to pump blood to the tissues and organs around the body. This is associated with a shift toward glycolytic metabolism, alterations in calcium handling contractility, disorganization of the sarcomere, mutations in genes coding for contractile proteins, loss of myocytes with fibrotic replacement, systolic or diastolic dysfunction, and “electrical remodelling" (i.e. alterations in the expression or function of ion-transporting proteins, or both) (see for references Hill and Olson, 2008). From a morphological point of view, cardiac growth may be classified in:

- *concentric remodelling*: increase in relative wall thickness with normal cardiac mass;
- *concentric hypertrophy*: increase in relative wall thickness and cardiac mass with little or no change in chamber volume;
- *eccentric hypertrophy*: increase in cardiac mass with increased chamber volume; relative wall thickness may be normal, decreased or increased.

Myocytes represent cardiac cells mostly involved in the remodelling process; other components include the interstitium, fibroblasts, collagen and coronary vasculature (Cohn et al., 2000). During cardiac hypetrophy, myocytes increase in size with changes in sarcomere organization, protein expression and activation of pro-growth signalling cascades. Cardiomyocytes plasticity is often accompanied by a re-induction of the "fetal gene program", in which patterns of gene expression mimic those seen during embryonic development (for references see Hill and Olson, 2008). Moreover, recent findings suggested the presence in the adult mammalian myocardium of a cell population with the behaviour and potential of cardiac stem cells (CSC): their presence has identified myocyte death and myocyte renewal as the two sides of the proverbial coin of cardiac homeostasis (Nadal-Ginard et al., 2003). Myocyte renewal depends on the differentiation of the CSCs into immature myocytes that divide two to four times before becoming terminally differentiated and permanently withdrawn from the cell cycle (Nadal-Ginard et al., 2003). Adult epicardial-derived (EDC) cells are also supposed to retain their stem cell capacity, being able to divide and differentiate into adult cardiac cells (fibroblasts, coronary vascular smooth muscle cells) (Winter and Gittenberger-de-Groot, 2007). In the presence of cardiac injury, increased workload or a number of bioactive molecules, this proliferation significantly contributes to pathological organ remodelling. These findings have opened an unexpected frontier toward the possibility to manipulate mammalian adult heart growth also for therapeutic benefit in human diseases. Identifying both stimuli and mechanisms which may switch on silent genetic and/or metabolic programs to allow adult mammalian cardiac cells to proliferate, is now the goal for modern cardiovascular research. A possible approach in this regard is to identify examples of cardiac plasticity among vertebrates. Cardiac flexibility is remarkably elevated in several non mammalian vertebrates like amphibians and fish. Accordingly, in last years fish and amphibian hearts have represented fundamental research tools to analyse molecular, cellular and tissue aspects of cardiac development, epigenetic remodelling and adult heart regeneration. In amphibians, adult ventricular myocytes retain their proliferative potential (Bettencourt-Dias et al., 2003). In fish, while myocytes hypertrophy significantly contributes to normal cardiac growth, the adult heart retains the ability for hyperplastic growth with age,

in response to changes in environmental conditions, increased demand associated with training/exercise activity and sexual maturation (Gamperl and Farrell 2004, and references therein; Cerra, et al., 2004). Extreme examples of this plasticity are provided by the complete epimorphic regeneration of newt (Bettencourt-Dias et al., 2003) and zebrafish (Poss et al., 2002) injuried ventricle. The increasing body of information on fish (i.e. zebrafish) cardiac plasticity suggests that molecular mechanisms, which are silent in the adult mammalian heart, are instead continuously active in these species, so that they drive, in response to appropriate stimuli, heart remodelling towards hyperplasia and/or hypertrophy.

Fish as experimental model to study physiological and patho-physiological cardiac plasticity

In recent years, fish have become widely used animal models to study at molecular, tissue, and organ level, morphological and functional cardiac modifications and their determinants. Although fish diverged from humans more than 400-million years ago, in a translational perspective, results obtained in fish may represent a significant tool to decipher the mechanisms involved in the mammalian cardiac morpho-functional remodelling. Fish provide a number of exceptional advantages. For example, laboratory fish models are small and can be bred and maintained in large numbers easily and at low cost. They offer the opportunity to combine the analytical clarity of developmental biology with the power of genetics. Moreover, transgenic lines can be easily and quickly produced. Fish are also amenable to high-throughput approaches such as whole-genome mutagenesis or chemical library drug screens (Schartl, 2014).

Among fish, the zebrafish (*Danio rerio*) represents the non-mammalian vertebrate model largely used to investigate molecular aspects of cardiac development and remodelling. Zebrafish shows all the advantages described above, e.g. small size, short generation time, facility in breeding in large numbers in the laboratory. Interestingly, it produces transparent eggs, which can be easily manipulated to study the embryonic development. Its genome is sequenced (Howe et al., 2013) and large genomic resources have been built up: transgenic fluorescent marker lines that allow these fish model to be used for bioimaging, downregulation of gene expression during early development (achieved by morpholinos) and gene knockout mediated

by genome-editing technologies. Zebrafish embryos are particular well suited for studying gene function, protein expression and cellular aspects of cardiovascular development (see for references Bakkers, 2011). Adult zebrafish represent a useful model to study heart regeneration. They are able to completely regenerate cardiac tissue after that ~20% of the heart is surgically removed from the apex (Poss et al., 2002). The site of injury initially clots off; this is followed by the replacement of red blood cells with fibrin. However, within the first month after injury, the fibrin is quickly replaced by cardiac myofibers, and by 2 months postinjury, the cardiac tissue results indistinguishable from the hearts of sham-operated controls (Poss et al., 2002). Three major regeneration events are proposed in the zebrafish heart: *i)* the epicardium is activated by re-expressing embryonic markers, contributing to new vascularization of the regenerating myocardial cells; *ii)* cardiomyocytes start to proliferate to replace lost myocardium; *iii)* the endocardium is also activated and starts retinoic acid synthesis, which supports cardiomyocyte proliferation (Bournele and Beis, 2016). Although several developmental signaling pathways have been functionally associated with zebrafish heart regeneration, molecular mechanisms involved are still unknown.

Besides zebrafish, other fish are now emerging as new experimental models useful as "evolutionary mutant models" for complex human diseases. This concept was first introduced by Craig Albertson and colleagues (Albertson et al., 2009), and it is based on the awareness that evolution by adaptation to a specific environment has resulted in species or populations of many animal groups, including fish, that exhibit phenotypes closely resembling or mimicking human diseases. Among others, specimens of cyprinidae (*Carassius carassius, Carassius auratus*), which routinely experience hypoxia during their lifecycle, represent useful models to study hypoxia tolerance and preconditioning mechanisms in mammals. Both the goldfish (*Carassius auratus*) and the crucian carp (*Carassius carassius*) are able to tolerate prolonged and severe hypoxic conditions and remain active when overwintering in ice-covered ponds (Bickler and Buck, 2007). This capacity has been correlated with their ability to generate ethanol as an anaerobic end product, which is acid–base neutral, in contrast with the normal glycolytic end product lactic acid. Ethanol is produced in the myotomal musculature and is released to the outside water through the gills (Bickler

and Buck, 2007). A remarkable feature of these species is their ability to maintain or increase cardiac performance and autonomic cardiovascular regulation in response to oxygen deprivation (Stecyk et al., 2004). In the anoxic crucian carp, the sustained cardiac output, the reduced peripheral vascular resistance and the conserved autonomic control ensure the rapid distribution of glucose to metabolically active tissues and the transport of waste lactate to the site of ethanol production (the muscle), thereby allowing for efficient ethanol shuttling to the gills for excretion (Stecyk et al., 2004). Similarly, the surprising capability of the goldfish heart to enhance its performance during acute hypoxia, as well as its hypoxia-enhanced sensitivity to the heterometric (i.e. Frank-Starling mechanism) regulation may be crucial for maintaining the functional and metabolic interactions between organs and tissues that are required for the hypoxia tolerance of the organism (Imbrogno et al., 2014).

Many fish species experience remarkable changes in cardiac mechanical performance associated, for instance, with thermal acclimation, or with changes in locomotive habits and/or body growth, or both (see for references Cerra et al., 2004; Imbrogno 2013), thus they represent appropriate models to evaluate the cardiac morpho-functional remodelling associated with body growth and changes in life style. This is exemplified by the European eel *Anguilla anguilla.* The eel has a complex life cycle which, following metamorphosis, includes a spawning migration requiring high levels of swimming performance and elevated metabolic demands (see for references Imbrogno, 2013). The relevant metabolic and cardio-respiratory capacities to satisfy tissue demands are associated to its great ability to regulate cardiac aerobic metabolism during hypoxia (McKenzie et al., 2003) and to the growth-related cardiac morphodinamic remodelling (Cerra et al., 2004). Indeed, by comparing small juvenile fish with their large adult counterparts, Cerra and colleagues (Cerra et al., 2004) demonstrated that, during ontogenetic growth, the eel heart undergoes morphodynamic changes. With respect to the small counterpart, the growing eel ventricle is characterized by enhanced hemodynamic performance expressed as a better capacity of the heart to sustain increased output pressure. These changes in mechanical behaviour positively correlate with structural modifications of the heart ventricle, consisting in an increase in *compacta* thickness and in the diameter of the

trabeculae in the *spongiosa*, together with a reduction of the lacunary spaces (Cerra et al. 2004). Therefore, because of these growth-related morphodynamic changes, the cardiac ventricle of small eels, with its limited response to pressure overload and large lacunary spaces, appears better suited to produce volume work; contrarily, the heart of the large eels is better adapted to produce pressure work (see for references Icardo et al., 2005).

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 cardiac plasticity in fish. Taking advantages of their experience in the field of fish cardiac physiology, the aim of this PhD research project was to analyse the influence of humoral and environmental factors on cardiac plasticity in fish. In particular, this thesis will provide an overview of results obtained by studies regarding the humoral control of cardiac remodelling mediated by chronic exposure to AngII in the heart of the European eel *Anguilla anguilla* and in the zebrafish *Danio rerio,* and the physiological and molecular mechanisms activated in the heart and in the muscle of the common carp *Carassius carassius* in response to hypoxia exposure. These two aspects will be separately analysed in Chapter 1 and Chapter 2, respectively. For each topic, a brief introduction on the state of art will be furnished.

During my PhD in *Life Science,* my research activity also contributed to obtain information about the role of neuropeptides and neuroendocrine modulators, such as Nesfatin-1, Chromogranin A-derived serpinine peptide, and Selenoprotein T, on the cardiac performance of the goldfish *C. auratus*. Published results will be not described in the thesis but attached as appendix in the pdf format.

CHAPTER 1

Humoral influences: role of AngiotensinII

1.1. INTRODUCTION

1.1.1. Teleost cardiac plasticity in response to humoral factors

To support the varying physiological requirements of the animal, the fish heart adjust its metabolic and hemodynamic functions in response to both extrinsic and intrinsic signalling pathways. The former represented by circulating and intracardiac hormones, the latter consisting of humoral autocoids (i.e. locally generated signalling substances). Both endocrine and autocoid signalings are responsible for beat-to-beat (e.g. Starling's law of the heart), short term (E-C coupling, myocardial contractility) and long-term (modified gene expression) cardiac modulation under both normal and stress-induced conditions.

Most of the knowledge on the morpho-functional heterogeneity and flexibility of the fish heart is based on studies on teleost fish. This group of fishes has been largely used to illustrate how endocrine circuits contribute to organ integration, in relation to the role of both classic [(i.e., catecholamines (CAT), angiotensin II (AngII) and natriuretic peptides (NPs)], and novel cardiac modulators [i.e., chromogranin-A (CgA) and its derived peptides]. Moreover, the cardiac role of locally released autacoids [i.e., carbon monoxide (CO), nitric oxide (NO) and hydrogen sulphide (H_2S)], which evolved as regulatory molecules before the establishment of hormonal networks typical of complex animal body plans, has been also deeply investigated (Gattuso et al., 2018).

A major integrative mechanism that contributes to teleost heart modulation by coordinating both humoral signals and the cross-talk between the endocardial endothelium (EE) and the myocardium, is represented by the nitric oxide synthase (NOS)/NO system. The comparatively greater amount of EE covering the internal cavity of the teleost heart represents an important source of NO that by acting as a freely diffusible messenger modulates the performance of the subjacent myocardium. This EE-NOS signalling, located at the crossroads of many extrinsic and intrinsic neuro-endocrine pathways, coordinates many chemically activated cascades. For example, endoluminal chemical stimuli such as acetilcholine (ACh), Ang II, vasostatin-1 (VS-1), catestatins (Cts), as well as β3 adrenoceptor activation, all converge in their contractile effects *via* NO signalling, which requires the obligatory

involvement of the EE (Imbrogno et al., 2001; Imbrogno et al., 2003; Imbrogno et al., 2004; Imbrogno et al., 2006; Imbrogno et al., 2010). Thus, the intracardiac NOS/NO system, located downstream and at the crossroads of a large number of regulatory endocrine pathways represents a "knot" for convergent molecular signalling. The result is the modulation of intracellular target, such as membrane ion channels, transporters and contractile proteins, and a fine control of myocardial contractility, relaxation and stretch induced modulation. As hereafter illustrated, this occurs also in the eel heart in which, results reported in this thesis documented the involvement of the NOS/NO system in mediating the cardiac effects of AngII.

1.1.2. Angiotensin II

Angiotensin II (AngII), the bioactive component of the Renin-Angiotensin System (RAS), is a pluripotent hormone whose biological actions are extensively studied in mammals. The RAS cascade starts with the renin-mediated cleavage of the decapeptide angiotensin I (AngI) from angiotensinogen. Then, a dipeptidyl-carboxypeptidase, the angiotensin converting enzyme (ACE), removes a C-terminal dipeptide from AngI, generating the octapeptide AngII. In mammals, the heart itself expresses all RAS components in both myocytes and fibroblasts (Dostal 2000 and references therein). In addition, in cardiac cells, the enzyme chymase contributes to AngI/II conversion.

A RAS, analogous to that found in mammals, is present in teleost fish (see Kobayashi and Takei, 1996). It was mainly studied in relation to ion and osmotic regulation, in particular under dehydrating conditions, such as seawater acclimation (references in Imbrogno and Cerra 2017). The bioactive peptide AngII has been identified and sequenced in various teleost species, including the chum salmon *Oncorhynchus keta* (Takemoto et al., 1983), the Japanese goosefish *Lophius litulon* (Hayashi et al., 1978) and the American eel *Anguilla rostrata* (Khosla et al., 1985).

The teleost heart possesses an intracardiac RAS. ACE activity is present in the ventricle of a variety of species (e.g. *Heteropneustes fossilis*, *Clarias batrachus, Channa gachua, Anabas testudineus, Notopterus chitala, Monopterus cuchia*: Olson et al., 1987), immunoreactive AngII-like material was described in the heart of the Antarctic teleost *Champsocephalus gunnari* (Masini et al., 1997), cardiac AngII binding sites were

detected in the trout *Oncorhynchus mykiss* (Cobb and Brown, 1992), and in the eel *A. anguilla* (Imbrogno et al., 2013). This suggests also in the teleost heart, the presence of an AngII-dependent intracardiac circuit of regulation.

In mammals, AngII-dependent cardiac effects are mediated by plasma membrane AT1 and AT2 receptors, classified according to the affinity for different antagonists (see references in De Gasparo, 2002, and Cerra et al., 2001). AT1 is mainly responsible for AngII-dependent positive myocardial inotropy and myocyte growth. It stimulates, via G-protein phospholipase C, Ca^{2+} mobilization, inositol triphosphate, diacylglycerol and protein kinase C (see Dostal, 2000, for references). It also activates the JAK-STAT pathway, thus inducing cardiac growth (Booz et al., 2002). In contrast, cardiac AT2 receptors, whose expression is reduced after birth, antagonizes AT1 growth promoting effects *via* a number of phosphatases. This receptor is also coupled with the NO/cGMP signalling, either directly or indirectly through enhanced bradykinin or endothelial NOS expression (references in Dostal 2000).

Several studies attempted to obtain the molecular and biochemical characterization of teleost AngII receptors (see for example Marsigliante et al., 1996; Tran van Chuoi et al., 1998). Studies were mainly performed by using commercial drugs and analogues with often negative or inconsistent results, possibly attributed to the lack of specificity of the drugs and/or intrinsic biochemical traits of the receptors (see Russell et al., 2001 for references). It was found that two AngII receptor types are present in the eel. One, characterized in the European eel *A. anguilla*, shows a cDNA sequence [GenBank accession number AJ05132 (Tran van Chuoi et al., 1998)] with 60% homology with the mammalian AT1 receptor (Russell et al., 2001); the other, characterized in the Japanese eel *Anguilla japonica* (Wong and Takei, 2013), shares the origin with mammalian AT2, as demonstrated by molecular phylogenetic and synteny analysis (Wong and Takei, 2013). Only AT receptors able to bind mammalian anti-AT2 antibody were detected by Western Blotting in the cardiac tissue of *A. anguilla* and were associated to the long-term effects of the peptide (Imbrogno et al., 2013).

1.1.3. AngII-dependent short-term modulation of cardiac activity

The heart of the American eel *A. rostrata* and the trout *O. mykiss* is either directly or indirectly stimulated by homologous [Asn1, Val5]-AngII (Oudit and Butler, 1995). Indirect effects are supposed to be mediated either by CAT or by adrenergic tone modulation. In fact, the dose-dependent increments in cardiac output (CO) and stroke volume (SV) observed in *O. mykiss* after the exposure to teleost AngII are inverted by α-adrenergic blockage (see for references Imbrogno and Cerra 2017). In contrast to these cardio-stimulatory effects, AngII administered to the isolated and *in vitro* perfused working eel (*A. anguilla*) heart reduces both inotropism and chronotropism. This direct cardio-inhibition involves AT1-like receptors, Gi/o proteins, the cholinergic system, and an EE–NO–cGMP–protein kinase G (PKG) cascade (Imbrogno et al., 2003). Although the reasons of this divergence are unclear, authors hypothesized a role of species-related differences and/or the organization level under study. This may be the case of results obtained on intact cardiovascular system *vs* those observed on *in situ* heart or on isolated and denervated working cardiac preparation. For example, in the *in vitro* heart of *A. anguilla* the AngII-dependent cardio-suppressive effect is suggested to function as a local mechanism of cardio-inhibitory protection that balances/opposes systemic cascades of convergent excitatory stimuli (Imbrogno et al., 2003). This effect, *in vivo* could be overridden by cardiovascular excitatory stimulation due to the synergism of adrenergic and RAS pathways, both activated under stress and emergency conditions (Hazon et al., 1995).

1.1.4. AngII-dependent long-term cardiac readjustment

In mammals, AngII is an important mediator of cardiac growth. By directly and indirectly cross-talking with growth-promoting and growth-inhibiting factors (i.e. NO, IGF-1, ET-1, NPs), and with angiogenic molecules (i.e. VEGF, FGF-1), AngII induces cardiac growth and remodelling, particularly under pathologic conditions (references in Imbrogno and Cerra, 2017).

Of note, also the teleost heart appears a target for AngII-induced long-term effects. In *A. anguilla*, chronic (4 weeks) administration of AngII elicits cardiac morpho-functional readjustment. AngII treated animals show an enhanced cardiac hemodynamic performance in response to afterload increases and this positively

correlates with structural modifications of the heart ventricle and/or with an incremented expression of proteins involved in the regulation of cell growth and apoptosis (Imbrogno et al., 2013). Of note, the application of CGP42112, a selective AT2 antagonist which cross-reacts with fish cardiac AngII receptors (Cerra et al., 2001; Imbrogno et al., 2003), revealed the involvement of this receptor subtype in the above-mentioned effects. This is of relevance since in mammals, AT2 generally offsets or opposes the AT1-induced actions on cell growth, blood pressure, and fluid intake, and mediates anti-growth and apoptotic actions (Gallinat et al., 2000). Contrarily, in teleost, this receptor appears to be associated to growth-promoting actions (Imbrogno et al., 2013). Moreover, AngII-treated hearts show an increased expression of a NOS recognised by mammalian anti eNOS isoform (eNOS-like). Since in mammals, a reduced eNOS-derived NO generation is responsible for hypertrophy and Reactive Oxygen Species (ROS) formation (Wenzel et al., 2007), it is possible that in teleost an AngII-dependent increase of NO bioavailability counter-balances the growth promoting signalling and contributes to coordinate cardio-protective programs (Imbrogno et al., 2013). The significance of the AngII-induced growth-promoting action on the eel in relation to cardiac and animal performance is unknown. However, this long-term effect must be considered in the context of the proliferative myocardial response of the piscine heart, which is opposite to the situation encountered in mammals. As exemplified by the complete regeneration of the zebrafish ventricle following cardiac injury (Poss et al., 2002), and the ontogenetic morpho-functional remodelling described in the eel (Cerra et al., 2004), in teleost, hypertrophy and hyperplasia represent the mechanisms of cardiac growth. It is possible that in fish the capacity to rapidly replace myocardial tissue has been retained during evolution as a function of the need for rapid cardiac growth during the adult phase. In fish, prolonged stimuli, e.g. those elicited by cold acclimation or chronic exercise, trigger proliferative signalling and hyperplastic and/or hypertrophic cardiac growth (Vornanen et al., 2005, and references therein). Thus in fish, differently from mammals, the compensatory proliferative signalling is not detrimental. Within this scenario, the long-term effects induced by neurohumoral agents, including AngII, may be fundamental components of the stress defence of the cardio-circulatory system of cold-blooded vertebrates (Imbrogno et al., 2013).

The influences of chronic (two months) AngII exposure on cardiac morpho-functional remodelling in fish has been investigated in the European eel *A. anguilla* and in the zebrafish *D. rerio*. As previously described, this two species show an elevated cardiac plasticity in response to physiological and pathophysiological stimuli, thus representing interesting animal models to evaluate structural and molecular heart remodelling associated to humoral influences.

Cardiac effects of AngII on the eel and the zebrafish heart will be described in the Sections A (AngII-dependent cardiac remodelling in the eel *A. anguilla*) and B (AngII-dependent cardiac remodelling in the zebrafish *D. rerio*), respectively. For each section, methods will be reported and results will be analysed and discussed.

SECTION A:

AngII-dependent cardiac remodelling in the eel A. anguilla

1.2A. MATERIALS AND METHODS

1.2A.1. Animals

Specimens of freshwater European eel (*A. anguilla*) weighing 83,31 ± 5,39 g (mean ± SD; n= 27), provided by a local hatchery, were kept in aerated freshwater at room temperature (18-20 °C) for 8 weeks and fed twice a week with commercial fish food. Animals were divided into two groups: the control and the AngII-treated group. According to Imbrogno and colleagues (2013), each animal has been subjected to intraperitoneal injections on alternate days (for 8 weeks) with:

- Control group (n=13): 1 ml of physiological saline.
- AngII-treated group (n=14): 1 nmol AngII gBW^{-1} in 1 ml of physiological saline.

Animal treatment with saline or AngII and heart samples collection were performed during 2013, i.e. before the new Italian law about the care and use of Laboratory Animals, effective from March 2014, in which the European eel is considered an endangered species.

1.2A.2. Chemicals

The homologue teleost octapeptide AngII (Oudit and Butler, 1995) was purchased from SIGMA. Solution was prepared in double-distilled water; dilutions were made in physiological saline immediately before use.

1.2A.3. Experimental protocols

1.2A.3.1. Morphological analysis

The hearts, removed from the pericardial cavity and flushed with phosphate buffered saline (PBS; pH: 7.6), were processed according to the following procedures: *Semithin sections*. The hearts from control and treated eels (n=3 for each condition) were fixed in 2.5% glutaraldehyde. Small cubes of tissue were taken from the middle anterior wall of the hearts. The pieces were dehydrated, embedded in EPON 812 (Shell Chemical Co., San Francisco, CA) and cutted (0.70 mm) with an ultramicrotome (Ultra Cut, Leica). Sections were stained with methylene blue, and observed with LEICA optical microscope (LEICA DM5000B). Images were

digitalized by using Adobe Photoshop 7.0 and the morphometrical evaluations (compacta thickness, percentage of muscular and vascular compartment) were carried out on images (10X magnification) using ImageJ 1.49v. Geometrical scaling was performed prior to start measurements. *Compacta* thickness was quantified by measuring the distance, from the border, between the epicardium and endocardium. The percentage of surface area occupied by empty spaces was calculated by thresholding on random images of different transverse and longitudinal ventricular sections. The resulting area (in pixels) was subtracted from the total area (in pixels) of the section, thus obtaining the overall area of myocardium and vascular components. The myocardial surface was obtained by subtracting from the overall area (myocardium plus vascular components) the vascularized area (percentage of area occupied by vessels), quantified by measuring each blood vessel (Adobe Photoshop CC15.2Portable).

Light microscopy. The hearts from control and AngII treated animals (eel: n=3 for each condition) were fixed in MAW (methanol:acetone:water, 2:2:1) and then dehydrated in graded ethanol, embedded in paraplast (Sherwood, St. Louis, MO, USA), and serially sectioned at 8 μm. The sections were placed onto Superfrost Plus slides (Menzel-Glaẗser, Braunschweig, Germany). Several sections were stained with either hematoxylin and eosin for a general assessment of tissue structure and with Sirius red for detection of collagen fibers. Slides were observed under a light microscope (Zeiss Axioscope); images were digitalized by Axiocam 105 color (Zeiss).

Immunofluorescence. The hearts fixed in MAW were dehydrated in graded ethanol, embedded in paraplast, and serially sectioned at 8 μm. Sections were rinsed in Tris-buffered saline (TBS) and incubated with 1.5% BSA in TBS for 1 h. They were then incubated overnight at 4 °C with rabbit polyclonal antibodies directed against NOSTRIN (cat# Sc-134803), AT2 receptor (cat# Sc-48452), and goat polyclonal anti pNOS3-Ser1177 (cat# Sc-12972), diluted 1:100 in TBS. All antibodies were from Santa Cruz Biotechnology, Inc., Heidelberg, German. For signal detection, after washing in TBS (3X10 min), slides were incubated with FITC-conjugated anti rabbit and anti-goat IgG (SIGMA, 1:100) and mounted with mounting medium (Vectashield, Vector Laboratories Burlingame, CA, USA). Slides were observed under a fluorescence microscope (Axioscope, Zeiss), and the images were digitalized by Axiocam 105

color (Zeiss). Negative controls were obtained on parallel sections treated in the same manner, excluding primary antibody. For nuclear counterstaining, sections were incubated with Propidium iodide (SIGMA; 1:10.000) for 5 min.

1.2A.3.2. Western blotting and densitometric analysis

To evaluate whether chronic AngII treatment affects the expression pattern of protein involved in cellular growth and NO metabolism, western blotting analysis was performed on heart extracts. The hearts of control and AngII-treated groups (n=3 for each condition) were rapidly immersed in liquid nitrogen and stored at -80 °C. The ventricle, separated from the atrium and the bulbus arteriosus, was prepared according to Amelio et al., 2006. Amounts of 60 μg of proteins were separated on 8% SDS-PAGE gel (for eNOS/peNOS detection) or 12% SDS-PAGE gels (for ERK1-2/ pERK1-2, NOSTRIN, Hsp90, Akt/pAkt, AT1 and AT2), and electro-blotted on to a nitrocellulose membrane. For immunodetection, blots were incubated overnight at 4°C with either rabbit polyclonal antibodies directed against NOSTRIN, Akt1/2/3 (cat# Sc-8312), pAkt1/2/3-Ser473 (cat# Sc-7985-R), ERK2 (cat# Sc-154), eNOS (cat# N3893), or mouse monoclonal antibodies directed against AT1 receptor (cat# Sc-57036), pERK1-2 (cat# Sc-7383), or goat polyclonal antibody directed against pNOS3-Ser1177, Hsp90 (cat# Sc-1055), AT2 receptor. eNOS was purchased from SIGMA; all other antibodies were from Santa Cruz Biotecnology.

Peroxidase linked secondary antibodies (anti-rabbit and anti- goat) (Amersham) were diluted 1:2000 in TBS-T containing 5% non-fat dry milk. Immunodetection was performed by using an enhanced chemiluminescence kit (ECL PLUS, Amersham). Autoradiographs were obtained by exposure to X-ray films (Hyperfilm ECL, Amersham).

Proteins were separated on 12% SDS-PAGE gels and electro-blotted on to a nitrocellulose membrane. For immunodetection, blots were incubated overnight at 4°C with mouse monoclonal antibodies directed against AT1 receptor or goat polyclonal antibody directed against AT2 receptor.

Immunoblots were digitalized and the densitometric analysis of the bands was carried out using WCIF ImageJ software based on 256 grey values (0=white; 256=black). Quantification of the bands was obtained by measuring (eight times on

each band) the mean optical density of a square area, after the background has been subtracted.

1.2A.3.3. Nitrite measurements

For nitrite measurements, hearts from control and AngII-treated eels (n=4 for each condition) were washed in a phosphate-buffered saline [50 mM phosphate buffer; pH 7.8; 85 mM NaCl; 2.4 mM KCl; 10 mM N-ethylmaleimide (NEM); 0.1 mM diethylenetriaminepentaacetic acid (DTPA)], and then dried on a paper towel, weighed and frozen in liquid N_2. Each heart was homogenized in 50 mM phosphate buffer (4 µl mg^{-1} tissue; pH 7.3), containing 10 mM NEM (N-ethylmaleimide) and 0.1 mM DTPA (diethyletriaminepentaacetic acid) to stabilize S-nitrosothiols. Samples were centrifuged (2 min, 16,000 g, 4°C) and the supernatant was immediately measured. Nitrite was measured by reductive chemiluminescence, using a Sievers (Boulder, CO, USA) NO Analyzer (NOA, model 280i) according procedures described in Hansen and Jensen 2010. Sample was injected into the NOA purge vessel, which contained a reducing agent that converted NO metabolites to NO. The NO was transported with a carrier gas (N_2) to the NOA reaction cell, where NO reacted with ozone to form O_2 and electronically excited nitrogen dioxide (NO_2*), which (on decay to its ground state) emitted light in the near-infrared region. The emitted light was detected by a photomultiplier, and the amplified signal was sent to a computer. An injected sample resulted in a peak, and the area under the peak was determined by integration. By comparing the area with areas produced by known nitrite standards, the amount of NO produced from the injected sample could be calculated.

The tri-iodide (I_3^-, made by adding NaI and I_2 to acetic acid) assay at 25°C was used to release NO from nitrite, SNO, FeNO and NNO compounds.

1.2A.4. Statistics

Differences in morphometric indices were expressed as means ± SEM of absolute values; statistic was assessed by unpaired t-test (*$p < 0.05$).

Compacta thickness and vascularization rate were calculated on 5 images for each group; values represent the means ± S.E.M. of 6 measurements for each image.

Statistical significance of differences was assessed using the Student's t-test (*$p < 0.05$; **$p < 0.005$).

For densitometric analyses, values were expressed as means ± S.E.M. of absolute values from individual experiments; statistic was assessed by unpaired t-test (**$p < 0.005$; ***$p < 0.0005$). GraphPad Prism software, version 4 (GraphPad Software, San Diego, CA) was used for all the statistical analysis.

1.3A. RESULTS

1.3A.1. Cardiac morphometry

Ventricular sections stained with Sirius red (Fig. 1A and B) showed collagen fibers localized both in the *compacta* and *spongiosa*. An increase of collagen fibers located around the sub-epicardial vessels, in coronary vessels wall, at the border between *compacta* and *spongiosa*, and in the interstitium of the *compacta* characterized the hearts of AngII-treated animals. Increased collagen fibers are also detected in the *spongiosa* layer at the level of subendocardium and within interstitial spaces (Fig. 1B).
Observations carried out on semithin sections (Fig. 1C and D) shown a significant increase of ventricle muscularity in treated eels, associated with an increased thickness of the *compacta* and trabecular size in the *spongiosa*. AngII-treated animals also showed an increased *compacta* vascular compartment (Fig. 1E, G, F).

1.3A.2. AngII receptors

Cardiac expression of AngII receptors, AT1 and AT2, has been evaluated by western blotting and immunofluorescence analysis by using mammalian anti-AT1 and AT2 antibodies.
AT1 receptor was undetectable by both immunoblotting and immunofluorescence. In contrast, densitometric analysis of the blot with anti-AT2 antibody revealed an increased expression of this receptor in AngII-treated hearts, with respect to the control (Fig. 2D). Immunofluorescence showed a different localization of AT2 in the hearts of control and treated animals. In particular, AT2 signal was revealed in the myocardiocytes of control hearts (Fig. 2A), and at the EE of AngII-treated hearts (Fig. 2B).

1.3A.3. ERK1-2

The ERK1-2 signalling pathway is involved in the regulation of myocardial proliferation and fibrosis (references in Mehta and Griendling, 2007). As revealed by western blotting analysis performed on cardiac extracts, prolonged exposition to AngII elicits a significant decrease of ERK1-2 phosphorylation in the hearts of AngII-

treated animals (Fig. 3A). This suggests a negative modulation of the ERK1-2 signalling in the chronic effects of AngII in the eel heart.

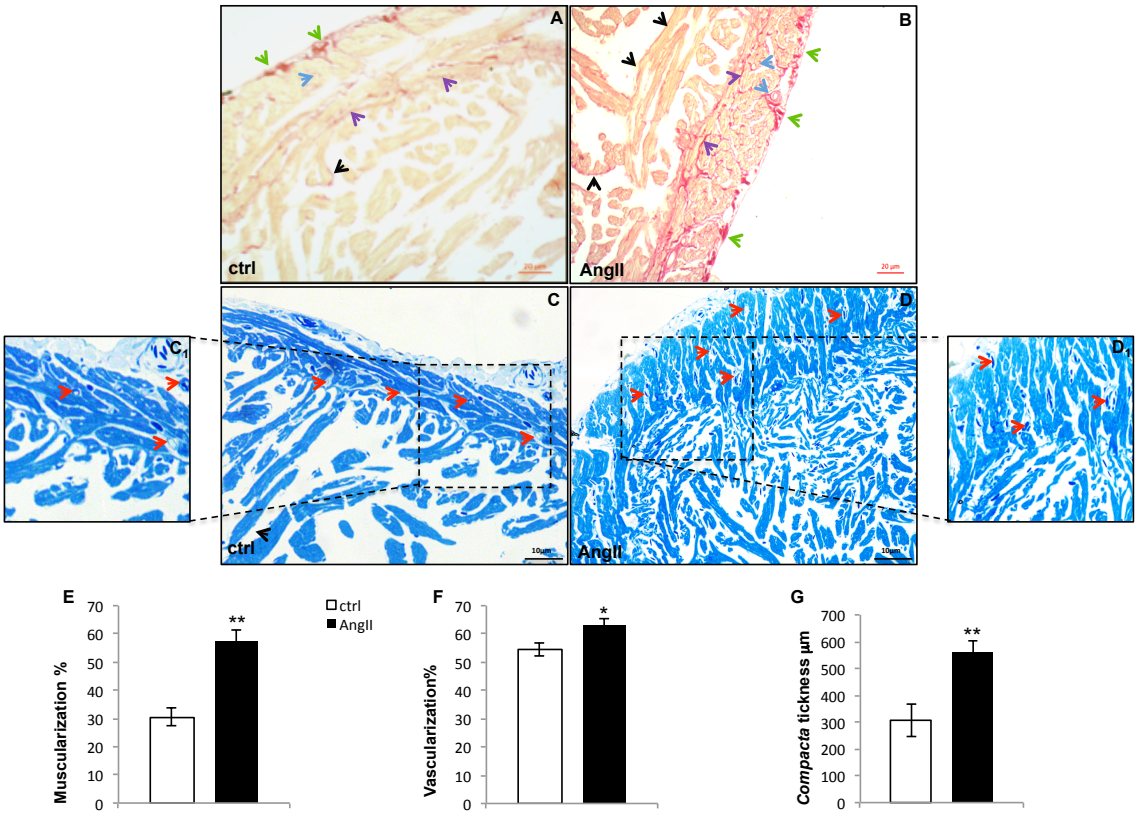

Fig. 1. Representative images showing morphological changes occurring in the eel ventricle after AngII treatment.

(A, B) Sirius red staining of control (A) and AngII (B) treated hearts. Collagen fibers localize at subepicardial level (green arrows), in the vessels wall (blue arrows), at subendocardial level (black arrows) and in the interstitium (violet arrows). (C, D) Methylene Blue stained ventricular cardiac semithin sections of control (C) and AngII (D) treated eels. After treatment, the increment of *compacta* thickness, and of muscular and vascular (red arrows) components is evident. Inset C1 and D1 represent higher magnification of the vascularized area of control and AngII treated hearts, respectively. Statistical significances are reported in the corresponding histograms (E, F, G) (*$p < 0.05$; **$p < 0.005$). Values represent the means ± SEM of 6 measurements for each image (N=5).

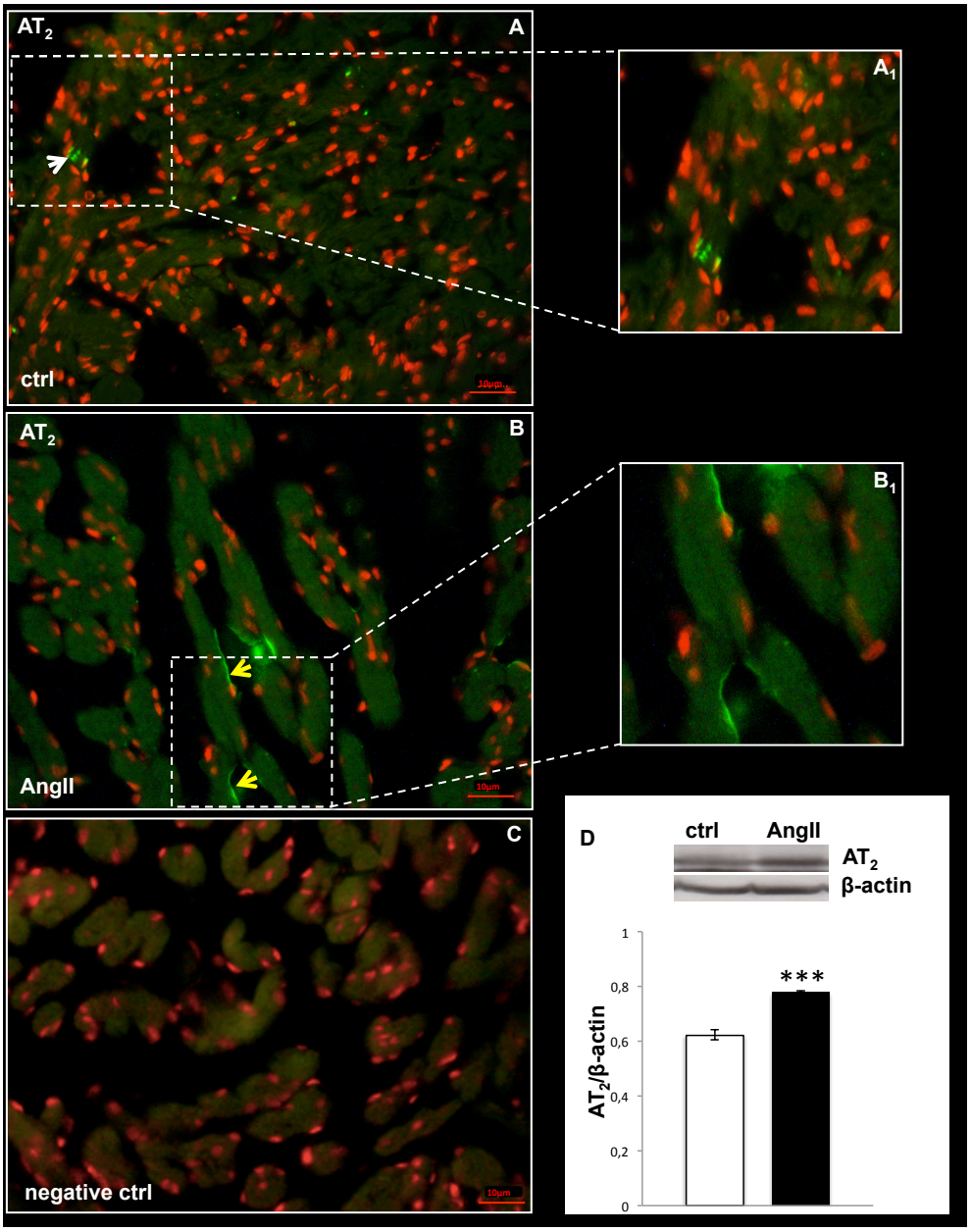

Fig. 2. AT2 immunolocalization and expression

(A, B) Representative images of AT2 immunolocalization in the ventricle of control (A) and AngII treated (B) eels. White arrow: myocardiocytes. Yellow arrows: endocardial endothelium. Nuclei counterstaining: propidium iodide. Inset A1 and B1 represent a higher magnification of AT2 positive signal. (C) Negative control. (D) Western blotting and densitometric analysis of AT2 in cardiac extracts of ctrl and AngII treated eels. In D, loaded protein amount was verified using anti-β-actin antibody. Statistical differences were evaluated by unpaired t-test (***$p < 0.0005$). Data are the means ± SEM of 3 determinations for each group.

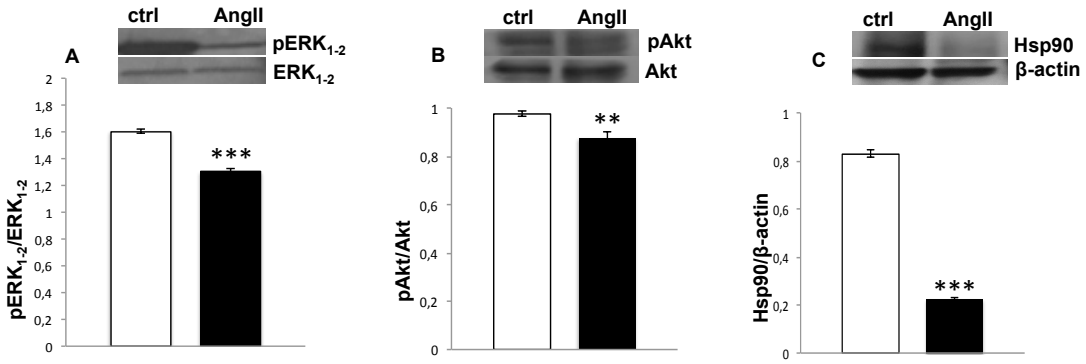

Fig. 3. Western blotting on cardiac extracts
Western blotting and densitometric analysis of pERK1-2/ERK1-2 (A), pAkt/Akt (B) and Hsp90 (C) in cardiac extracts of control and AngII-treated eels. In C, loaded protein amount was verified using anti-β-actin antibody. Statistical differences were evaluated by unpaired t-test (**p < 0.005; ***p < 0.0005). Data are the means ± SEM of 3 determinations for each group.

1.3A.4. NOS/NO system

To evaluate the influence of AngII treatment on the cardiac NO production, the concentration of nitrite [used as an index of constitutive NOS activity (Hansen and Jensen 2010)] was determined in heart homogenates of both control and AngII-treated eels. Results showed a significant decrease of nitrite concentration in hearts from treated animals compared with the control group (control: 1.33 ± 0.13 mmol L^{-1}; AngII-treated: 0.60 ± 0.04 mmol L^{-1}).

Western blotting of ventricular extracts revealed no significant differences in eNOS phosphorylation between control and treated hearts (data not shown) accompanied by a slight decrease of pAkt expression (a positive modulator of NOS) in treated animals (Fig. 3B). However, immunofluorescence analysis showed a different localization of peNOS in the heart of control and treated animals. In particular, while in the control hearts, a peNOS signal was captured at the EE level and in the cardiomyocytes of ventricle (Fig. 4A) and atrium (Fig. 4B), in treated hearts the enzyme prevalently localized in the myocardiocytes (Fig. 4D and E); only a weak

signal appeared on the EE. In addition, at vascular level, a reduction of the peNOS signal (Fig. 4G) was observed in the vascular endothelium of treated animals, with respect to the control (Fig. 4F).

In line with the reduction of pAkt expression, western blotting analysis revealed a strong decrease in the expression levels of Hsp90, a positive modulator of NOS, after AngII treatment (Fig. 3C). These results are accompanied by a significant increase in the expression of the eNOS disabling protein NOSTRIN (Fig. 5D), a negative modulators of eNOS activity which promotes the translocation of the enzyme from the plasma membrane to intracellular vesicles (Michel and Vanhoutte 2010, and references therein). Although in both untreated (Fig. 5A) and AngII-treated (Fig. 5B) hearts, immunofluorescence localized NOSTRIN on the vascular endothelium of the greatest vessels (Fig. 5C) and on the ventricular EE, a strongest signal was detected at the endocardial level in AngII treated hearts (Fig. 5B).

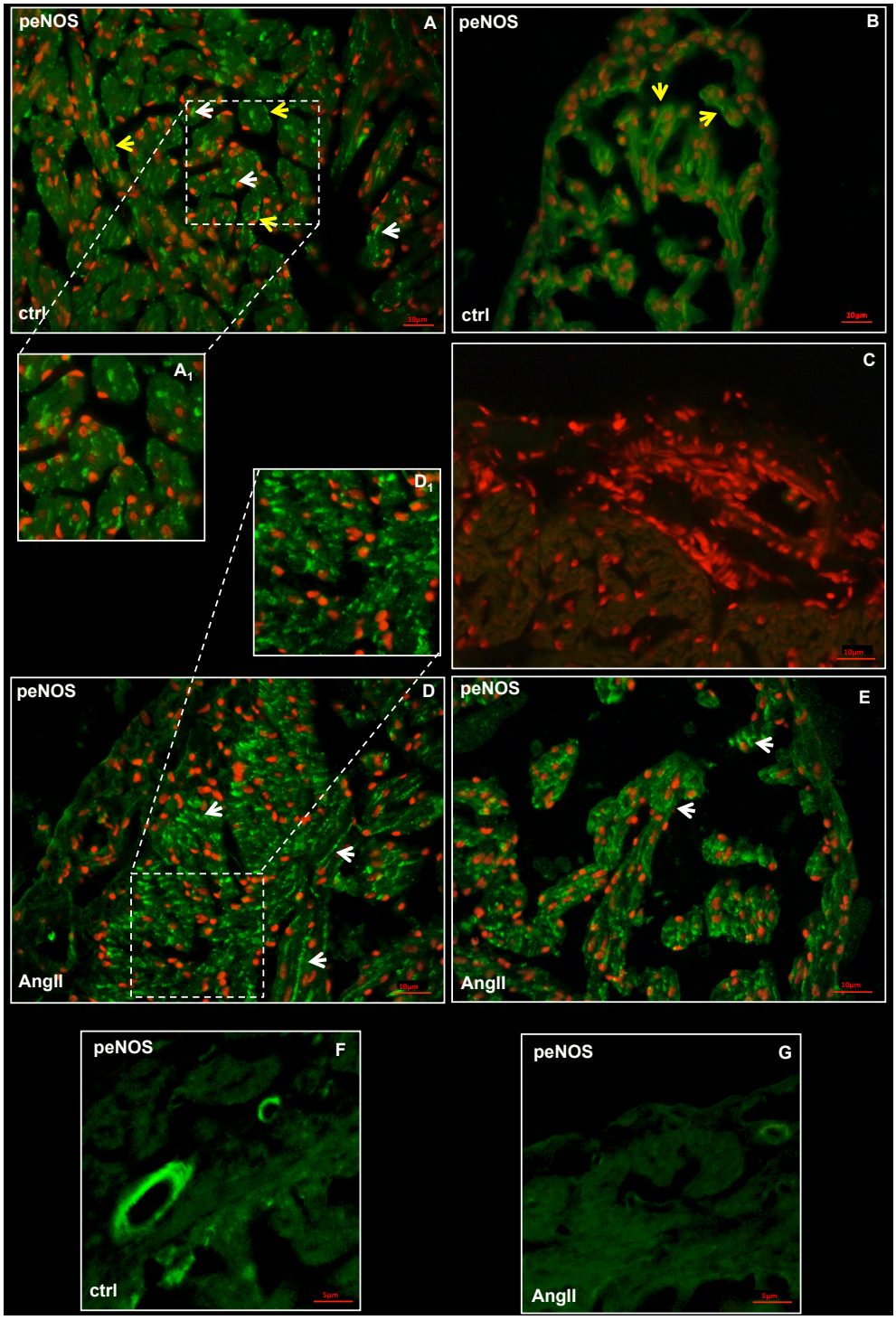

Fig. 4. peNOS immunolocalization
Representative images showing peNOS immunolocalization on cardiac sections of control (A, B, F) and AngII (D, E, G) treated eels. (A, D) ventricle, (B, E) atrium, (F,G) vessels. Inset A1 and D1 represent a higher magnification of peNOS positive signal. (C) Negative control. White arrows: myocardiocytes. Yellow arrows: endocardial endothelium. Nuclei counterstaining: propidium iodide.

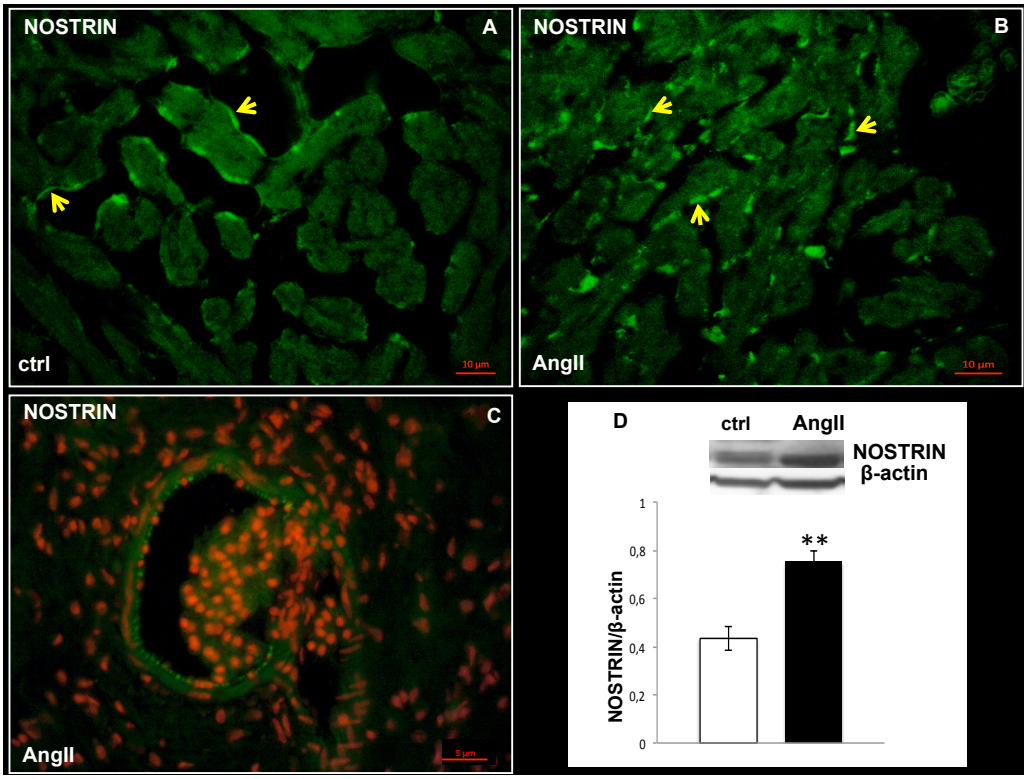

Fig. 5. NOSTRIN immunolocalization and expression

Representative images of NOSTRIN immunolocalization in ventricular sections of control (A) and AngII-treated (B, C) eels. NOSTRIN localized in endocardial (yellow arrows) and vascular endothelium (red arrows). C: nuclear counterstaining with propidium iodide. (D) Western blotting of NOSTRIN in cardiac extracts of control and AngII treated eels. Loaded protein amount was verified using anti-β-actin antibody. Statistical differences were evaluated by unpaired t-test (**$p < 0.005$). Data are the means ± SEM of 3 determinations for each group.

1.4A. DISCUSSION

The results reported in this section showed, for the first time, that chronic (two months) treatment with AngII induces structural modifications of eel ventricular wall and myocardial vessels. This remodelling was paralleled by modification of NO production, and by changes in the expression and localization of molecules which regulate NOS activity, such as the protein kinase B (Akt), the eNOS disabling protein NOSTRIN, and the heat shock protein 90 (Hsp90).

With respect to the compact type of ventricular myoarchitecture typical of homeotherm hearts, eel shows a mixed type ventricle myoarchitecture with an outer *compacta* layer that enclose an inner *spongiosa* made up of a crisscrossed array of myocardial bundles (trabeculae) (see Cerra et al., 2004, for references). As revealed by morphological analyses, the cardiac ventricle of eels chronically exposed to AngII undergoes structural modifications, becoming more "muscularized" than untreated animals. This remodelling occurs at the level of both *compacta* and *spongiosa*. In particular, when compared to control animals, AngII-treated eels showed an increased *compacta* thickness, and a larger diameter of the trabeculae that form the *spongiosa*. These changes are accompanied by higher collagen deposition in the compact layer, shown by an intense Sirius red staining, particularly localized in the wall of subepicardial and coronary vessels. Enhancement of the compact myocardium is a strategy of cardiac growth that occurs in many fish species [*Ciprinus carpio*: (Bass et al., 1973); *Salmo salar*: (Poupa et al., 1974); *Thunnus thynnus*: (Poupa et al., 1981); *Salmo gairdneri*: (Farrell et al., 1988)]. It was described in *A. anguilla* during ontogenetic growth (Cerra et al., 2004) and in response to one month exposure to AngII (Imbrogno et al., 2013). Of note, we observed in eels treated for two months with AngII that the increment of the compact layer is accompanied by an increased vascularisation. In non-mammalian vertebrates, the growing heart increases capillarization of the compact myocardium through tightly controlled local mechanisms, which include mechanical (e.g. myocardial stretch), metabolic and growth factors such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) (Tomanek and Ratajska 1977, and references therein). It is conceivable that the enlarged vascular supply, which occurs in the *compacta* of

the AngII-treated hearts, may allow the heart to cope with the metabolic and energetic demands of the deeper myocardial cells.

In mammals, AngII-dependent effects on myocyte growth correlate with the activation of the AT1 receptor (see Booz et al 2002, for references). Contrarily, the AT2 receptor, is generally reported to mediate anti-growth and apoptotic actions (Gallinat et al., 2000). However, a role for AT2 receptors in AngII-dependent cardiomyocyte hypertrophy and cardiac fibrosis was described in mice from Ichihara and co-workers (Ichihara et al., 2001). In *A. anguilla*, the few available data correlate the growth-promoting actions of AngII to the activation of a receptor able to bind mammalian anti-AT2 antibody (Imbrogno et al., 2013). AT1 receptors were not detected in control and AngII-treated eels. Conversely, western blotting and immunofluorescence showed, in the heart of the two groups of animals, a different expression and spatial localization of AT2 receptors. In fact, while in control hearts AT2 receptors are expressed in few myocardiocytes, after AngII treatment, they localize more widely in the EE. This suggests that, contrarily to mammals, in the eel heart AT2 receptors participate to the mechanisms of remodelling induced by AngII. No data about a putative AngII-dependent modulation of ATs expression are available in fish. Also in mammals the molecular mechanisms responsible for the expression of the AT2-R gene are not fully defined, although an upregulation of the AT2 expression has been observed in different experimental models, also in response to AngII exposition, and in relation to various physio-pathological conditions (references in Lemarie and Schiffrin, 2010). It is interesting to underline that, although eel AT2 shares its origin with mammalian AT2, it underwent a peculiar evolution after divergence of its lineage (Wong and Takey, 2013). This may explain the contrasting features showed by this receptor in mammals *vs* teleost, as for example the activation of different transduction pathways (Wong and Takey, 2013), and thus the different elicited responses.

Several studies in mammals reported that AngII exerts its actions on the heart by recruiting ERK1-2 (see for references Mehta and Griendling, 2007). This kinase is known for its stimulatory role in the mechanisms of myocardial proliferation and

fibrosis (Aoky et al., 2006; Bueno et al., 2000). However, more recent experiments performed on *in vivo* knock-out mice showed that ERK1-2 signalling is not required for mediating cardiac hypertrophy (Purcell et al., 2007). In mammalian cardiomyocytes, by acting on AT1 and/or AT2-receptor, AngII exerts distinct effects on ERK1-2 activity. Through AT1 receptor it may induce ERK1-2 activation, while it may inhibit ERK1-2-dependent pathway through AT2 receptor (Wei et al., 2000). Consistent with these observations, the significant reduction of the activated (phosphorylated) form of ERK1-2 observed in AngII-treated animals, is accompanied by an increased expression of AT2 receptors, suggesting that ERK1-2 down-regulation is mediated by the AngII-AT2 binding.

An important aspect of the molecular network guiding the AngII-dependent cardiac morpho-functional modulation is the cross-talk with the NO signalling. In mammals, in addition to the AT2-mediated control of NO production (references in Dostal, 2000), AngII activates a number of signal-transduction pathways that result in reduced NO levels (Morawietz et al., 2006). In turn, NO can counteract AngII-dependent effects by down-regulating the synthesis of both ACE and AT1 receptors (Wiemer et al., 2001; Takemoto et al., 1997). Thus, NO generation is both downstream and upstream the AngII-dependent cascade. NO is a vital signalling molecule that exerts its physiological effects by reversible binding/reacting with hemes, thiols or amines, forming iron-nitrosyl (FeNO), S-nitroso (SNO) and N-nitroso (NNO) compounds (Hill et al., 2010). Furthermore, NO is short-lived and excess NO is rapidly oxidized to nitrite and nitrate. Tissue levels of nitrite represent a marker of constitutive NOS activity and consequently of NO generation (Hansen and Jensen, 2010). By measuring total nitrite concentration in endothelial cell cultures, Li and co-workers observed an AngII-dependent reduction of eNOS-dependent NO production (Li et al., 2016). In this study, we found a significant decrease in the ventricle nitrite concentration from 1.33 mmol L^{-1} in control animals (which compares with other fish species (Sandvik et al., 2012; Jensen et al., 2015) to 0.6 mmol L^{-1} in AngII treated eels. This suggests that chronic exposure to AngII is accompanied by a reduced NO generation, possibly mediated by a blunted NOS functionality. To analyse this possibility, the molecular modulation of NOS-dependent NO production

has been analysed. A variety of regulatory proteins cooperate to finely control eNOS activity. This is the case of calmodulin, Hsp90 and protein kinase B (Akt), that positively modulate eNOS (Fulton et al., 1999). At the same time, negative eNOS modulators include caveolin, and NOSTRIN (Feron and Balligand, 2006; Zimmermann et al., 2002). The latter represents a crucial eNOS trafficking controller that, by binding the enzyme, triggers its translocation from the plasmalemma to vesicle-like subcellular structures, thus blunting eNOS-dependent NO production (Zimmermann et al., 2002; Su, 2014). In teleosts, as well as in agnathans and chondrichthyans, a canonical eNOS seems to be absent (Andreakis et al., 2011). Nevertheless, physio-pharmacological approaches, as well as NADPH-diaphorase and immunolocalization with heterologous mammalian antibodies, revealed the presence of an "eNOS-like" activity in the heart of several teleost species (Imbrogno et al., 2011; Amelio et al., 2006; Amelio et al., 2008; Imbrogno et al., 2016). In the present study, by western blotting and immunofluorescence, we analysed whether and to which extent, the reduced NO generation observed in the eel heart after chronic exposure to AngII is associated with a modulation of eNOS controlling proteins, Akt, Hsp90 and NOSTRIN. Compared to the control, AngII-treated hearts showed comparable expression of peNOS-like enzyme, a slight decrease of p-Akt and a high decrement of Hsp90. Akt-mediated activation of eNOS is strongly influenced by the association between Hsp90 and the enzyme (Brouet et al., 2001). In fact, Hsp90 can either directly act as a scaffold factor between eNOS and Akt, or indirectly prevent Akt dephosphorylation from protein phosphatase 2A (Sato et al, 2000). In addition, decreased levels of Hsp90 are responsible of a reduced displacement of caveolin-1 from eNOS (Sessa, 2004), blunting enzyme activity. The bio-molecular results obtained on the remodelled heart of AngII-treated eels suggest that long-time exposure to the hormone may induce a decrease of eNOS-like activity by modulating the expression of its intracellular regulators Akt and Hsp90. Of note, in AngII-treated hearts, we also found an enhancement of ventricular NOSTRIN expression accompanied by changes in its localization. On ventricular sections of both untreated and treated eels, NOSTRIN localizes on the vascular endothelium of greater vessels and on the EE. In particular, in treated eels, the EE is characterized by a higher NOSTRIN signal and a reduced peNOS localization. This suggests that, as in

mammals (see Dudzinski and Michel, 2007 for references), also in the eel heart a higher NOSTRIN expression is responsible for a reduction of NO production through enzyme inactivation.

Of note, a reduction of peNOS signal that resemble those occurring on endocardial cells was observed also in large vessels of chronic AngII treated animals. Several evidences exist in mammals on the relationship between AngII and the NOS/NO system on both normal and pathological vascular homeostasis. It was shown in mammals that AngII induces an increase in endothelial oxidative stress, with subsequent adverse effects on vascular function and a down-regulation of eNOS activity (see Galougahi et al., 2014 for references). Thus, eNOS modifications induced by AngII affect the downstream NO activated signalling pathways with effects on the vascular tone. As shown in mammalian aortic rings, AngII decreases endothelium-dependent relaxation, and this in part contributes to endothelial dysfunction. Based on these observations it is possible to speculate that in the eel heart, chronic Ang-II treatment, by reducing vascular NO generation may negatively affect vascular relaxation, and thus the perfusion of the compact myocardium where larger vessels are mainly located.

SECTION B:

AngII-dependent cardiac remodelling in the zebrafish D. rerio

1.2B. MATERIALS AND METHODS

1.2B.1. Animals

Wild type, 6-10 month-old adult zebrafish (*Danio rerio*) were obtained from a local commercial supplier and maintained at the Department of Biological and Environmental Sciences and Technologies, ate the University of Salento (Lecce, Italy). Fish maintenance conditions were set according to Westerfield (1995). Briefly, fish were kept in plastic tanks containing deionized water (pH 7.2-7.4) at 28 °C on 14 h light/10 h dark cycle, under constant mechanical and chemical filtration, and fed thrice daily with commercial fish food. During treatments, specimens of adult zebrafish weighing 0,61 ± 0,04 g (mean ± SD; n= 14) were divided into two groups (i.e. the untreated control and the AngII-treated group). AngII-treated animals were exposed to waterborne AngII 500 nM according to Kumai et al. (2014), for 8 weeks. The peptide was administered every 3 days, 10 min after water change and before feeding. At the endpoint, animals were anesthetized with tricaine methanesulfonate (MS222, SIGMA-Aldrich Chemical Co., UK) before experimental analysis. Animal care and procedures were in accordance with the U.S. National Institutes of Health's Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1996) and with Directive 2010/63/EU.

1.2B.2. Chemicals

The homologue teleost octapeptide AngII (Oudit and Butler, 1995) was purchased from SIGMA. Stock solution (0.1 mg/mL) was prepared in ultrapure water; dilutions to 500 nM were directly made in the water for treatment.

1.2B.2.1. AngII octapeptide conjugation with Quantum Rods (QRs)

Conjugation of the AngII octapeptide by covalent binding to fluorescent nanocrystals (Quantum rods, QRs) was performed by Dr. L. Blasi and A. Quarta (CNR-Nanotech Institute, Lecce, Italy), according to optimized protocols. In brief, QRs were synthesized as previously described by Carbone and collaborators (2007). Then, QRs were made hydrophylic by polymeric coating with amphiphilic Poly(maleic anhydride-alt-1-octadecene) according to Di Corato et al. (2008). The polymer

structural groups represent docking points for functionalization by polyethylene glycol (PEG) and conjugation with peptides. In particular, PEG functionalization reduces the non-specific adhesion, while enhances immune-mimicking of QR nanoparticles (Quarta et al., 2012). Functionalization and subsequent conjugation were obtained by the EDC/NHS (1 - ethyl - 3(3 - dimethylaminopropyl) carbodiimide/N - hydroxysuccinimide) technique. The conjugation reaction of the AngII octapeptide was performed for 3 hours at R.T. by mixing the peptide with equal volumes of QRs (1 μM), PEG (500 μM) and EDC/NHS (50 mM). The synthesis flowchart is described in Fig. 6. Effectiveness of conjugation has been confirmed by agarose gel electrophoresis and photoluminescence assays.

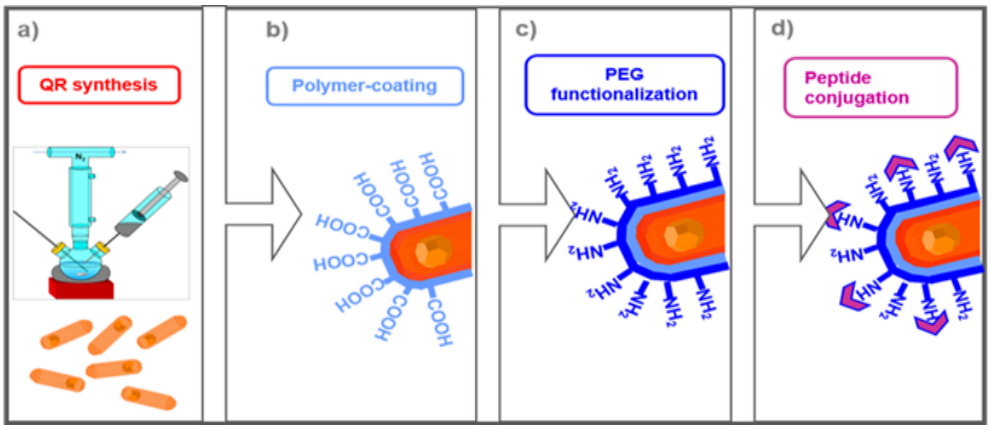

Fig. 6 QR-AngII synthesis flowchart.
Coating, functionalization and conjugation steps.

1.2B.3. Experimental protocols

1.2B.3.1. Morphometric analysis

Basic measurements, including body weight, body length, heart weight and ventricle length of each animal were performed as here following specified. Under anaesthesia, each animal was weighted and body length manually measured with a millimeter ruler, from the tip of the mouth to the body/caudal fin juncture. Isolated hearts were weighted, and images were acquired by a digital camera (Nikon AZ-100 stereomicroscope, implemented with the Nikon NIS-Elements D-suite software).

ImageJ software was used to measure heart length. Cardiosomatic index has been obtained from direct measurements [CSI = 100 x (heart weight/body weight)].

1.2B.3.2. Heart rate

Heart rate was calculated by direct observation, under a dissecting microscope, and by counting for three times the number of beats per minute.

1.2B.3.3. Morphological analysis

To evaluate tissues involved in waterborne AngII intake, fish exposed to the fluorescent peptide (QR-AngII) were entirely fixed in MAW and then dehydrated in graded ethanol, embedded in paraplast, and serially sectioned at 8 μm.

Hearts from control and AngII treated zebrafish (n=3 for each condition) were fixed in MAW and subjected to light microscopy. For technical details see Materials and Methods in section A.

1.2B.3.4. Western blotting and densitometric analysis

Hearts from adult zebrafish were isolated and stored in RNALater (Ambion). They were then processed for protein extraction by using the AllPrep DNA/RNA/Protein mini kit (Cat No./ID: 80004, Qiagen) protocol and reagents, according to the manufacturer's instructions. Total heart tissue lysis was performed with the AllPrep lysis buffer by using a mini homogenizer in 1.5 mL sterile tubes. At the end of the extraction protocol, protein extracts were stored at -20°C.

Western blotting was performed as previously described (Materials and Methods of section A). Proteins were separated on 12% SDS-PAGE gels and electro-blotted on to a nitrocellulose membrane. For immunodetection, blots were incubated overnight at 4°C with mouse monoclonal antibodies directed against AT1 receptor (1:1000) or goat polyclonal antibody directed against AT2 receptor (1:1000).

Immunoblots were digitalized and the densitometric analysis of the bands was carried out using WCIF ImageJ software as described in Materials and Methods of section A.

1.2B.4. Statistics

Differences in morphometric indices were expressed as means ± SEM of absolute values; statistic was assessed by unpaired t-test (*$p < 0.05$).

For densitometric analyses, values were expressed as means ± SEM of absolute values from individual experiments; statistic was assessed by unpaired t-test (***$p < 0.0005$).

1.3B. RESULTS

1.3B.1. Cardiac morphometry and morphology

Somatic and cardiac morphometric parameters are used to provide indirect information on growth, nutrition, and overall health status for both individuals and populations (Vargas and Vàsquez, 2016). In particular, in adult healthy zebrafish, cardiosomatic parameters are used as reference values to assess the influence of environmental and humoral factors on cardiac function and morphology (Vargas and Vàsquez, 2016). Accordingly, these values are here used to evaluate the effects of chronic AngII exposure on zebrafish body and heart morphology. Results are summarized in Table 1.

Group	Body length (cm)	Body weight (g)	Heart weight (mg)	Ventricle length (mm)	CSI (%)
Control	3.03 ± 0.047	0.56 ± 0.04	0.82 ± 0.08	1.60 ± 0.17	0,17 ± 0,01
AngII-treated	3.03 ± 0.030	0.59 ± 0.05	1.50 ± 0.20*	1.62 ± 0.07	0,23 ± 0,02*

Table 1. **Effects of chronic AngII exposure on zebrafish body and heart morphology**. Values are the mean±SEM of absolute values (N=8). Statistic was assessed by unpaired t-test (*$p < 0.05$).

Measured values in both control and AngII treated animals resulted comparable with those proposed by Vargas and Vàsquez for adult healthy zebrafish, suggesting that experimental conditions did not affect the health status of the animal. No significant differences have been observed in fish body weight and length, not in the average ventricle length between control and AngII treated animals. Of note, in AngII treated animals, a slight but significant increase in the heart weight and in the relative Cardiosomatic index (CSI) has been evidenced (*$p<0,05$; N=8). Deviations from an average CSI implies an increased or decreased heart mass, which may indicate a cardiac ventricular remodelling (Hu et al., 2000). In our preparation, the increase in heart weight and CSI is accompanied by structural differences in ventricular wall of AngII treated animals with respect to the control group. In particular, heart sections of treated zebrafish stained with hematoxylin and eosin (Fig. 7A and B) showed

increased ventricular muscularity associated to an increased *compacta* thickness and a reduction of lacunary spaces. In both control and AngII-treated animals, Sirius red staining localized collagen fibers at the subendocardial level of both *compacta* and *spongiosa* (Fig. 7C and D). However, a strong increase of the collagen fibers amount has been observed after AngII treatment.

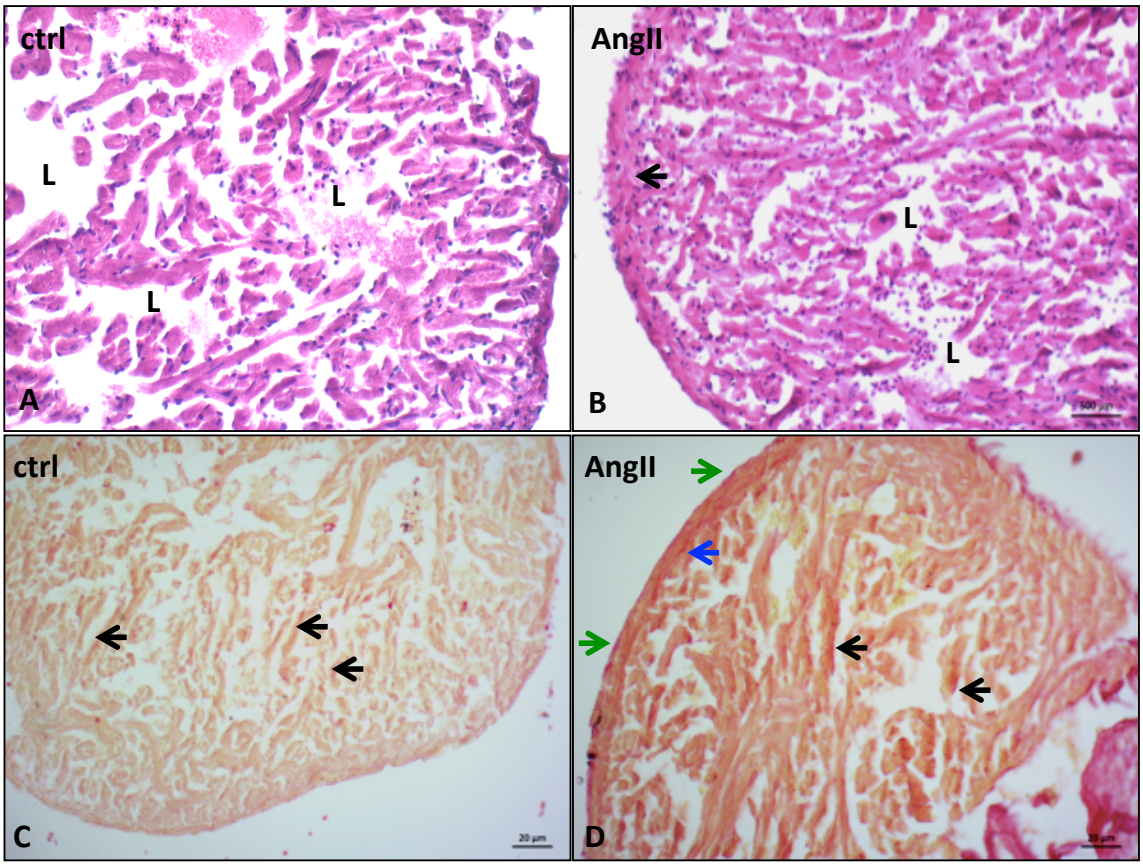

Fig. 7. Representative images showing morphological changes occurring in the zebrafish ventricle after AngII exposure.
(A, B) Hematoxylin and eosin staining of control (A) and AngII (B) treated hearts. After treatment, the increment of *compacta* thickness (black arrow) and of muscular component, associated to a reduction of lacunary spaces (L), is evident. (C, D) Sirius red staining of control (C) and AngII (D) treated hearts. Collagen fibers (in red) localize at *compacta* level (green arrows), at subendocardial level of both *compacta* and *spongiosa* (black arrows) and at the border between the two layers (blue arrow).

1.3B.2. Heart rate

In fish, the response of heart rate to AngII short-term exposure varies among species. In the Antarctic fish *Pagothenia borchrevinki*, heart rate decreased in response to AngII (Axelsson et al. 1994), while in rainbow trout, a significant increase of heart rate has been reported following intracerebroventricular AngII administration (Le Mevel et al. 1994). In the eel *A. anguilla*, intravenous injections of AngII elicited a significant increase in heart rate, which possible involves catecholamines (Oudit and Butler, 1995). A direct chronotropic effect in response to AngII has been described by Fuentes and Eddy (1998) on the trout heart *in vivo*. No information is available in fish about the influence of chronic exposure to AngII on heart rate. The present study demonstrates that in zebrafish, chronic AngII treatment elicits a significant increase in heart rate (control: 112 ± 8.3 beat min^{-1}; AngII-treated: 141 ± 8,7 beat min^{-1}; $p<0,05$; N=8). If this increase is related to a direct chronotropic effect of the peptide on the zebrafish heart or to the morphological rearrangement observed, needs to be further investigated.

1.3B.3. AngII receptors

In order to investigate the type of AngII receptors involved in the structural remodelling of the zebrafish heart, cardiac expression of both AT1 and AT2 has been evaluated by western blotting analysis by using mammalian anti-AT1 and AT2 antibodies.

Immunoreactive bands corresponding to the molecular weight of AT1 and AT2 were detected in cardiac extracts of both control and AngII treated animals. Densitometric analysis of the blot revealed a significant increased expression of both receptors in AngII-treated hearts, with respect to the control (Fig. 8). The involvement of these two receptors in the modulation of cardiac remodelling is still dubious. In mammals, despite AngII-dependent effects on myocyte growth correlate with the activation of the AT1 receptor (see Booz et al., 2002 for references), but not of AT2, Ichihara and co-workers (2001) reported in mice a role for AT2 receptors in AngII-dependent cardiomyocyte hypertrophy and cardiac fibrosis. In fish, the few available data suggests an involvement of the AT2 receptor in the AngII-dependent cardiac remodelling in the eel *A. anguilla* (Imbrogno et al., 2013). In this study, the AngII-

dependent cardiac effects involve both receptors. Whether in the zebrafish heart the transduction pathways activated modulate positively or, as in mammals, in opposite way cardiac growth needs to be further investigated.

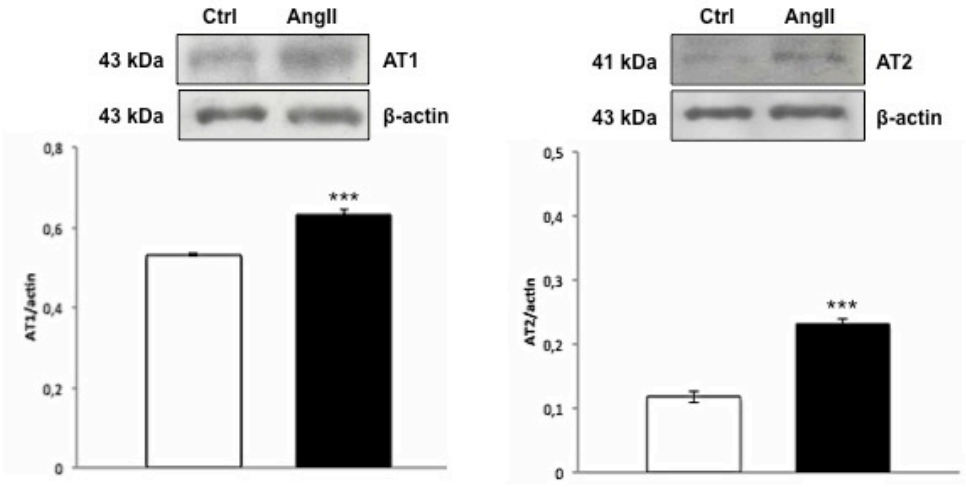

Fig. 8. AT receptors expression on cardiac extracts
Western blotting and densitometric analysis of AT1 and AT2 in cardiac extracts of control and AngII-treated zebrafish. Loaded protein amount was verified using anti-β-actin antibody. Statistical differences were evaluated by unpaired t-test (***$p < 0.0005$). Data are the means ± SEM of 3 determinations for each group

1.3B.4. Work in progress

Experiments are actually in progress to clarify the molecular mechanisms involved in the AngII-dependent morpho-functional remodelling of adult zebrafish heart. In particular, the ongoing research is aimed to:

1. identify tissues involved in the AngII waterborne intake;
2. deeper investigate structural remodelling of the ventricle wall (ultrastructural analysis);
3. identify molecular pathways involved in cardiac remodelling, including the putative role of the cardiac NOS/NO system (western blotting and real-time PCR).

To evaluate tissues involved in the AngII waterborne intake (point 1), an experimental protocol has been already set up. In collaboration with Dr. L. Blasi and A. Quarta (CNR-Nanotech Institute, Lecce, Italy), the piscine homologues AngII peptide has been conjugated to a fluorophore (QRs, see section 2.2.1). Zebrafish were exposed to the fluorophore alone (QR), or to the fluorescent peptide (QR-AngII) for 1 week. After the treatment, fish have been entirely processed for immunofluorescence. Very preliminary results suggest a role of gut and skin in peptide waterborne uptake. In fact, as shown in Fig. 9, strong fluorescent signals are captured in the intestinal folds and at the subepidermal level of zebrafish exposed to AngII-QR. Weak signals were captured in the gut and skin of animals exposed to the fluorophore alone. These data are in agreement with Sun and colleagues, which suggested that adult zebrafish can absorb small molecules dissolved in water through their skin, gills or by water intake (Sun et al., 2009).

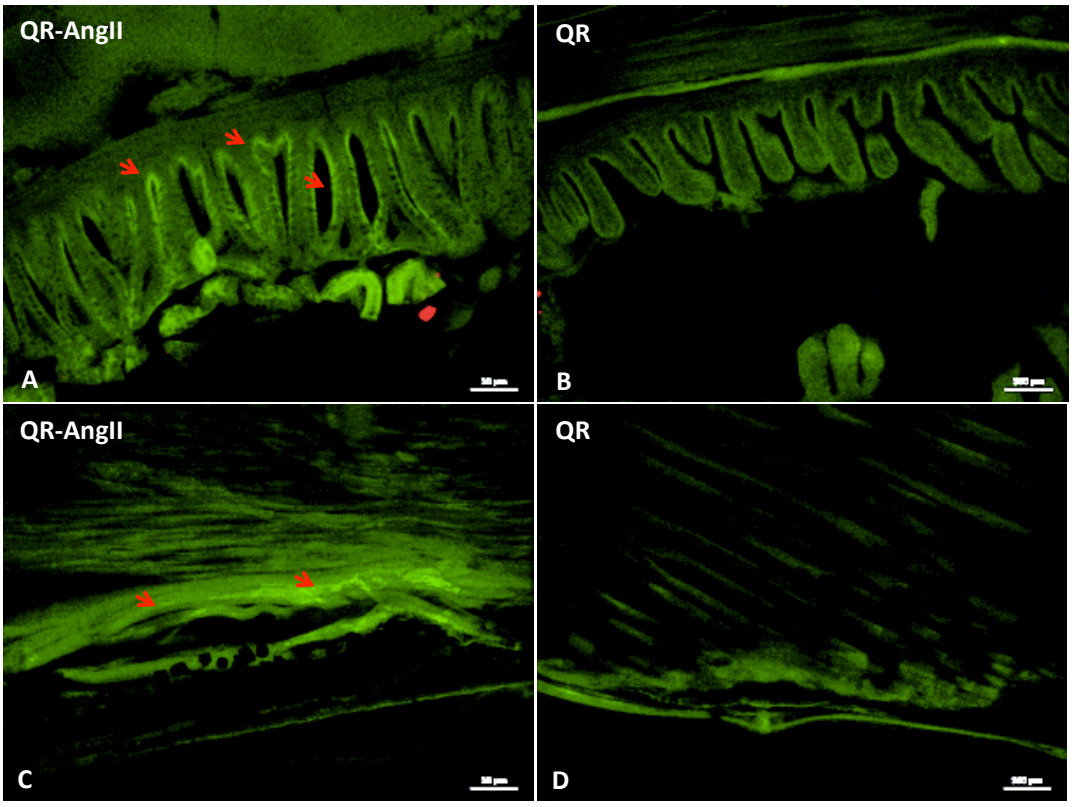

Fig. 9. Representative images showing immunolocalization of QR and QR-AngII in zebrafish.
Immunolocalization of QR-AngII (A,C) and QR (B,D) in the gut (A,B) and skin (C,D) of zebrafish exposed to the fluorescent AngII (QR-AngII) or to the fluorophore alone (QR).

1.5. CONCLUSIONS CHAPTER 1

Results reported in this first part of the thesis provide large evidence that both the eel and the zebrafish heart undergoes cardiac remodelling if exposed for two months to the cardioactive hormone AngII. In the eel, this remodelling occurs through molecular mechanisms which involves modification of NO production, and changes in the expression and localization of molecules which regulate NOS activity, pointing to the cardiac NOS/NO system as an important coordinator/integrator of molecular signal cascades which control cardiac form and function.

Future research will contribute to better decipher the complex molecular neuro-humoral networks which control cardiac remodelling in fish. In this context, the use of animal models, such as the eel, characterized by a remarkable morpho-functional adaptability to a variety of environmental factors (temperature, partial pressure of oxygen, pH, environmental pollutants, etc.) (Imbrogno 2013), as well as the zebrafish which shows a marked cardiac regenerative capacity, and several advantages including genome knowledge and easy genes manipulation (Bournele and Beis, 2016), could be of help.

CHAPTER 2

Hypoxia influences: the role of the NOS/NO system

2.1. INTRODUCTION

2.1.1. Hypoxia tolerance in fish

Molecular oxygen (O_2) represents an essential gas for life. Insufficient O_2 supply leads to tissue hypoxia which often results in organ damage. A major target of hypoxia is the heart, in which hypoxia can induce changes in muscle metabolism, reactivation of fetal gene programs and hypertrophy responses, modifications in extracellular matrix composition and mitochondrial biogenesis, as well as in the expression of intracellular effectors (i.e. the NO pathway, hypoxamiRs, HIF, etc.) (Fago and Jensen, 2015).

When compared to terrestrial animals, water-breathing organisms are much more likely to be exposed to wider temporal and spatial variations of O_2 supply. This is largely due to the inherent properties of the water and to the rapid fluctuations in the pattern of O_2 production and consumption (Nikinmaa et al., 2011). Several animal species are adapted to tolerate regular and often severe hypoxia. This is the case of various fish, frog, and turtle species that tolerate anoxia, and some snakes and insects that can endure severe hypoxia (Hermes-Lima and Zenteno-Savin, 2002). Teleost fish exhibit a very large spectrum of O_2 sensitivity, moving from species showing an extraordinary ability to tolerate hypoxia and anoxia to species that dramatically suffer O_2 deprivation. Among teleost fish, examples of hypoxia/anoxia resistance are the members of the cyprinid genus Carassius, such as the goldfish (*Carassius auratus*), and the crucian carp (*Carassius carassius*), which exhibit a striking capacity to survive and remain active for long periods under low O_2, even tolerating anoxia (Bickler and Buck, 2007). This capacity is correlated with the ability to generate ethanol as anaerobic end-product, which is acid–base neutral, in contrast with the normal glycolytic end-product lactic acid. Among cyprinids, the zebrafish (*Danio rerio*) is characterized by a growth-dependent transition from hypoxia tolerance to sensitivity (Padilla and Roth, 2001). Since its genome is fully sequenced it is of benefit for studying the components of hypoxia-resistance pathways in fish.

Despite the different abilities shown by vertebrates to tolerate a limited O_2 availability in the environment or in internal tissues, many studies performed in both mammalian and non-mammalian animal models (see Fago and Jensen, 2015, for

references) suggest a common set of concerted physiological responses. They basically include depression of O_2 consumption rates, protection against oxidative damage and, at least in air-breathing species, redistribution of blood flow into the circulation. All these responses require the activation of a complex network of intracellular cascades, such as those related to NO and its metabolites. Thus NO represents a common signalling molecule able to control and coordinate the molecular circuits which sustain adaptive hypoxia-dependent physiological responses (Fago and Jensen, 2015).

2.1.2. The NOS/NO system

NO is generated by the family of NOS isoenzymes [i.e. the constitutive endothelial (eNOS) and neuronal (nNOS), and the inducible (iNOS), isoforms], which convert L-arginine into L-citrulline and NO, in the presence of O_2 and NADPH as essential cofactors. This reaction, because of the obligatory requirement for molecular O_2, is vulnerable to hypoxia (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). NO can also react with O_2, yielding peroxynitrite ($ONOO^-$) (Ronson et al., 1999), and this depletes the bioactivity of the gas (Guzik et al., 2002). All reactive nitrogen species are responsible for protein post-translational modifications because of either ability to induce either S-nitrosation [the formation of a covalent bond between an NO^+ equivalent and a nucleophilic centre (amine or thiol)], or S-nitrosylation [the addition of NO without changing the formal charge of the substrate (metal centre or radical species)] (Heinrich et al., 2013). An uncontrolled nitrosation/nitrosylation of cysteine residues may induce nitrosative stress, with important effects on proteins activity, stability, conformation and/or ability to interact with other molecules (Foster et al., 2009). In the presence of a reduced O_2 availability, when the conversion of L-arginine in L-citrulline and NO is compromised, nitrite can be reduced back to NO, providing an alternative pathway for gas generation (Gladwin et al., 2005; Lundberg et al.,

2009). This NO regeneration occurs through acidic disproportionation and enzymatic reduction via Xanthine Oxidoreductase (XOR), mitochondrial enzymes, or deoxygenated Hb, Mb and neuroglobin (see for references Hansen and Jensen, 2010). Also nitrate contributes to NO homeostasis since it can be slowly reduced to nitrite by the ubiquitous enzyme XOR (Jansson et al., 2008). The nitrate-nitrite-NO pathway may be considered complementary to the classical L-arginine-NOS pathway. All these pathways partly work in parallel, but when O_2 availability is reduced and NOS activity is decreased, nitrite reduction to NO becomes more pronounced.

A large number of studies, performed mainly on mammals during the last two decades, have recognized the critical role of NO and its metabolites, in particular nitrite and S-nitrosothiols (SNO) in the mechanisms which control the cardiac response to low O_2 availability. This role has been recently extended also to non mammalian vertebrates, including fish, in which a growing body of evidence has documented the cardioprotective role of NO and its related nitrosative signal, under hypoxic challenges.

It is well established in fish that, if the NOS activity is compromised by limited O_2, an increased NOS expression or, alternatively, a nitrite reduction to NO, stabilize NO levels, and this contributes to protect the hypoxic myocardium (Hansen and Jensen, 2010; Sandvik et al., 2012; Imbrogno et al., 2014).
Experimental evidences indicating the NO involvement in cardiac homeostasis of teleosts fish under hypoxic/anoxic conditions mainly derive from studies on the goldfish *C. auratus*, a champion of hypoxia tolerance. In the goldfish heart, 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 importantly contributing to maintain fish myocardial function in the presence of hypoxia or anoxia (Stecyk et al., 2004). In line with these observations, Imbrogno and colleagues (2014) showed that, during acute hypoxia, the goldfish heart enhances its basal performance, as well as the sensitivity to heterometric (i.e. Frank-Starling) regulation. This has been considered an important mechanism for maintaining functional and metabolic interactions between organs

and tissues, required for the hypoxia tolerance of the organism. Interestingly, in the goldfish, exposure to hypoxia is accompanied by an increased myocardial NOS expression, pointing to 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 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 the hypoxia inducible factor (HIF)-1α (Imbrogno et al., 2014) which activates a number of critical genes (including NOS) that contribute to cell survival (Ong and Hausenloy, 2012).

As above illustrated, under hypoxic conditions, a significant source of NO is represented by nitrite. Compared to terrestrial animals, fish are exposed to an additional direct uptake of exogenous nitrite from the environmental water across the respiratory surfaces (Jensen, 2009). This external nitrite supply is an important source for the internal NO generation during severe hypoxia. An example is the crucian carp that, when exposed to deep hypoxia, takes up ambient nitrite across the gills and directs it to tissues, including the heart (Hansen et al., 2016). Moreover, hypoxia-tolerant species, such as the goldfish and the crucian carp, possess an intrinsic ability to increase intracellular nitrite concentration and nitrosylated compounds during deep hypoxia and anoxia in tissues with high myoglobin and mitochondria content, such as the heart (Sandvik et al., 2012; Jensen et al., 2014). This occurs at the expenses of extracellular nitrite. The extracellular-intracellular transfer of nitrite is facilitated by nitrite binding to intracellular proteins that, by keeping low the cytosolic concentration of free nitrite, allows inward diffusion (Hansen and Jensen, 2010).

2.2. MATERIALS AND METHODS

2.2.1. Animals

Crucian carp, *Carassius carassius* (Linnaeus 1758), of mixed sex, weighing 45.4±1.85 g (mean±S.E.M.; N=42) were caught in a local pond (Langsted, Funen, Denmark) in July and transferred to two 200 L holding tanks, where pond water was gradually changed to experimental water (Odense tap water mixed with demineralized water in a 1:4 ratio). The fish stayed in the tanks for 17 days and were fed with commercial trout pellets (Inicio, Biomar, Denmark), while being acclimated to 15°C and a 12 h: 12h light:dark cycle in normoxic (PO_2>140 mmHg) water. Normoxia was obtained by bubbling air, and water was exchanged daily.

The fish were subsequently moved to four normoxic experimental aquaria (100 L, with 10–11 fish in each) for 5 days without feeding; 60 L of water was exchanged twice daily. Two aquaria were maintained normoxic (PO_2>140mmHg) for one additional day, while the two other aquaria were bubbled with N2 for 1 day to expose the fish to deep hypoxia (1<PO_2<3 mmHg). The water surface was covered with expanded polystyrene, and deep hypoxia was reached within 4 h. Water PO_2 was measured using an optical Hach Lange optode (HQ 40d, Loveland, CO, USA). Water nitrite concentration stayed below 0.5 $\mu molL^{-1}$ and water Cl^- concentration was 260 $\mu molL^{-1}$.

Fish were caught individually and anesthetized in 2% MS222 (ethyl-3-aminobenzoate methanesulfonate) dissolved in experimental water. The fish were weighed and blood was taken from the caudal vessels, after which tissues were dissected out in the following order: heart, liver, white and red muscle. Blood was centrifuged (2 min, 8000 g, 15°C) and plasma was separated. Tissues were rinsed in phosphate-buffered saline [50 $mmolL^{-1}$ phosphate buffer pH 7.8; 85 $mmolL^{-1}$ NaCl; 2.4 $mmolL^{-1}$ KCl; 10 $mmolL^{-1}$ N-ethylmaleimide (NEM); 0.1 $mmolL^{-1}$diethylenetriaminepentaacetic acid (DTPA)], dried on tissue paper and weighed. All samples were snap frozen in liquid nitrogen and stored at −80°C until further analysis.

The procedures and experiments were conducted in accordance with Danish laws on animal experimentation. All chemicals were purchased from Sigma-Aldrich (Steinheim, Germany).

2.2.2. Nitrate reduction and determination of NO metabolites

To measure tissue nitrate reductase activity, subsamples of tissues weighing between 19 and 200 mg (depending on the tissue) were thawed and homogenized in 3 times their weight of phosphate buffer (100 $mmolL^{-1}$, pH 7.0) and 1 vol % protease inhibitor (P2714, Sigma-Aldrich). Heart and liver tissues were homogenized using zirconium oxide beads (ZROB05, 5 mm, Next Advance, NY, USA) in a Bullet Blender Blue (Next Advance) and muscle tissue was homogenized with a tissue grinder (Struers, Heidoloph, Denmark). Homogenates were centrifuged (10 min, 500 g, 4°C), and supernatants were decanted and divided into subsamples that were snap frozen, or used for determination of protein concentration. Final concentrations of protein (7 $mg\ ml^{-1}$), cofactors (1 $mmolL^{-1}$ NADPH, 500 $\mu molL^{-1}$ NADH, 500 $\mu molL^{-1}$ NAD^+, 500 $\mu molL^{-1}$ GSH) and nitrate (300 $\mu molL^{-1}$) were achieved by mixing appropriate amounts of buffer (250 $mmolL^{-1}$ sucrose, 10 $mmolL^{-1}$Tris-HCl, pH 7.0), homogenate, cofactor mix and nitrate in the given order. The volume of buffer was adjusted to fit the final volume in incubations with or without nitrate. Immediately after mixing, one aliquot was used to determine NO metabolites at time zero and the other subsample was subsequently bubbled with pure nitrogen for 2 min (heart and liver) or 7 min (muscle), sealed with Parafilm and incubated for 5 h at 25°C before a further determination of NO metabolites. To ascertain a persistent N2 atmosphere, we also performed experiments with liver homogenates in shaking Eschweiler (Kiel, Germany) tonometers receiving a continuous flow of humidified N2. The role of xanthine oxidoreductase (XOR) in nitrate reduction was examined by adding the XOR inhibitor allopurinol at 2 $mmolL^{-1}$. Nitrite and nitros(yl)ated compounds were determined by chemiluminescence (NO analyzers: Model CLD 77 AM, Eco Physics, Duernten, Switzerland; and Model 280i, Sievers, Boulder, CO, USA), as previously described (Yang et al., 2003; Hansen and Jensen, 2010). Nitrate was assessed with a vanadium chloride assay, and nitrite and nitros(yl)ation compounds (SNO+FeNO+NNO) were determined in a triiodide assay. To distinguish between nitrite and [SNO]+[FeNO]+[NNO], the samples were treated with sulfanilamide (Hansen and Jensen, 2010). NO-metabolites were calculated as absolute concentrations in $\mu molL^{-1}$ assuming a tissue density of 1 $kg\ L^{-1}$.

2.2.3. Muscle Mb concentration

Mb concentration was determined spectrophotometrically using the method developed by Reynafarje (1963) with small modifications (Helbo and Fago, 2012). White muscle (~200 mg) and red muscle (~100 mg) were homogenized in 4.25 and 9.25 times their mass of hypotonic buffer (40 mmol l−1 KHPO4, pH 6.60), respectively. We used a knife homogenizer (ultra-turrax T25, IKA-labortechnic, Staufen, Germany) on ice, 3 times 30 s with and 30 s break in between, to avoid heating up the homogenates. After centrifugation (50 min, 15.000 g, 4°C), homogenates containing Mb were equilibrated with CO for 3 min and ~0.001 g dithionite was added to reduce any ferric heme (Helbo and Fago, 2012). Absorbance spectra were collected from 700 to 400 nm, and Mb concentration (mg g^{-1} wet mass) was determined from the difference in absorbance at 538 and 568 nm [which is zero for carboxyhemoglobin (HbCO) but not for carboxymyoglobin (MbCO)], using reported extinction coefficients for the corresponding dilution (Reynafarje, 1963).

2.2.4. Ethanol

Ethanol was determined in plasma and white muscle homogenates using a commercial ethanol assay kit (MAK076, Sigma-Aldrich) according to the manufacturer's instructions. Ethanol concentration was measured by a SpectraMax Plus Microplate Reader (Molecular Devices, Sunnyvale, CA, USA).

2.2.5. Statistics

Graphing and statistical analyses were performed in Prism 5.0 (GraphPad Software, San Diego, CA, USA) and Origin 8.5 (OriginLab Corporation, Northampton, MA, USA). Results are presented as means±s.e.m. and statistical differences between means were evaluated using unpaired t-test, one-way analysis of variance (ANOVA) with Bonferroni post hoc test or two-way ANOVA, as appropriate. Furthermore, linear regression and one- sample t-test (to compare means with zero) were used. We applied a significance level of $P<0.05$ and data that did not fulfil the assumption of equal variances (Bartlett's test) were log-transformed prior to analysis.

2.3. RESULTS

2.3.1. NO metabolites and Mb levels in tissues exposed to deep hypoxia

Crucian carp exposed for 1 day to deep hypoxia showed slightly lower plasma nitrite concentration (Fig. 10A) and plasma [SNO+ FeNO+NNO] (Fig. 10B) compared with normoxic fish. Plasma nitrate concentration (Fig. 10C) did not differ between normoxic and deeply hypoxic groups.

In red skeletal muscle, nitrite concentration increased to more than double the normoxic value after exposure for 1 day to deep hypoxia, whereas it stayed constant in white skeletal muscle at a value that was similar to that in normoxic red muscle (Fig. 11A). The rise in red muscle nitrite concentration during deep hypoxia was paralleled by a significant increase in the concentration of nitros(yl)ated compounds (SNO+FeNO+NNO; Fig. 11B). In white muscle, [SNO+FeNO+NNO] did not change (Fig. 11B). A plot of all individual muscle [SNO+FeNO+NNO] values against corresponding nitrite concentrations revealed a highly significant (R^2=0.811) linear increase in [SNO+FeNO+NNO] with increasing nitrite concentration (Fig. 11D). Tissue nitrate concentration was slightly higher in white than in red muscle but only decreased non-significantly during deep hypoxia (Fig. 11C).

The level of Mb was significantly higher in red muscle than in white muscle but was unaffected by the level of ambient oxygen (Fig. 12A). Hence, red muscle Mb was 3.68±0.369 mg g^{-1} (corresponding to 0.22 mmolL^{-1}) in normoxic fish and 2.98± 0.362 mg g^{-1} in deeply hypoxic fish, which is 7–8 times higher than in white muscle, where Mb was 0.499±0.104 mg g^{-1} in normoxic fish and 0.361±0.075 mg g^{-1} in deeply hypoxic fish. When connected values of muscle nitrite and muscle Mb concentration from all individual fish are plotted against each other, it is evident that there is no linear correlation between muscle nitrite and Mb concentration, either when combining all groups or when analysing them independently (Fig. 12B). The main difference is that nitrite levels in deeply hypoxic red muscle are raised above the values in the other groups (Fig. 12B).

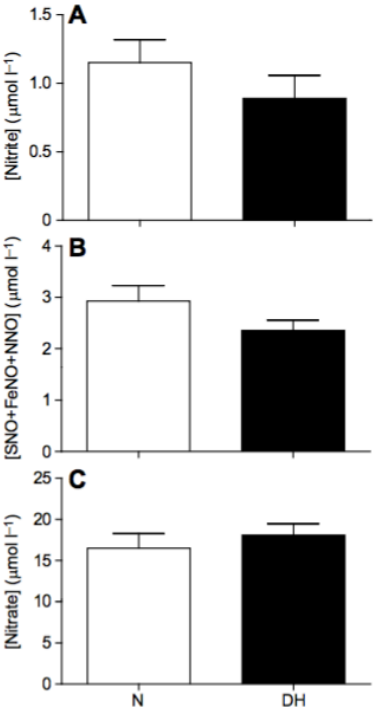

Fig. 10. Plasma NO metabolites in normoxic and deeply hypoxic crucian carp.
Concentrations of nitrite (A), S-nitroso (SNO)+iron-nitrosyl (FeNO)+N-nitroso (NNO) compounds (B) and nitrate (C) in plasma of crucian carp after 1 day exposure to normoxia (N; PO2>140 mmHg) or deep hypoxia (DH; 1<PO2<3 mmHg). Values are means±s.e.m. (N=15 for each group).

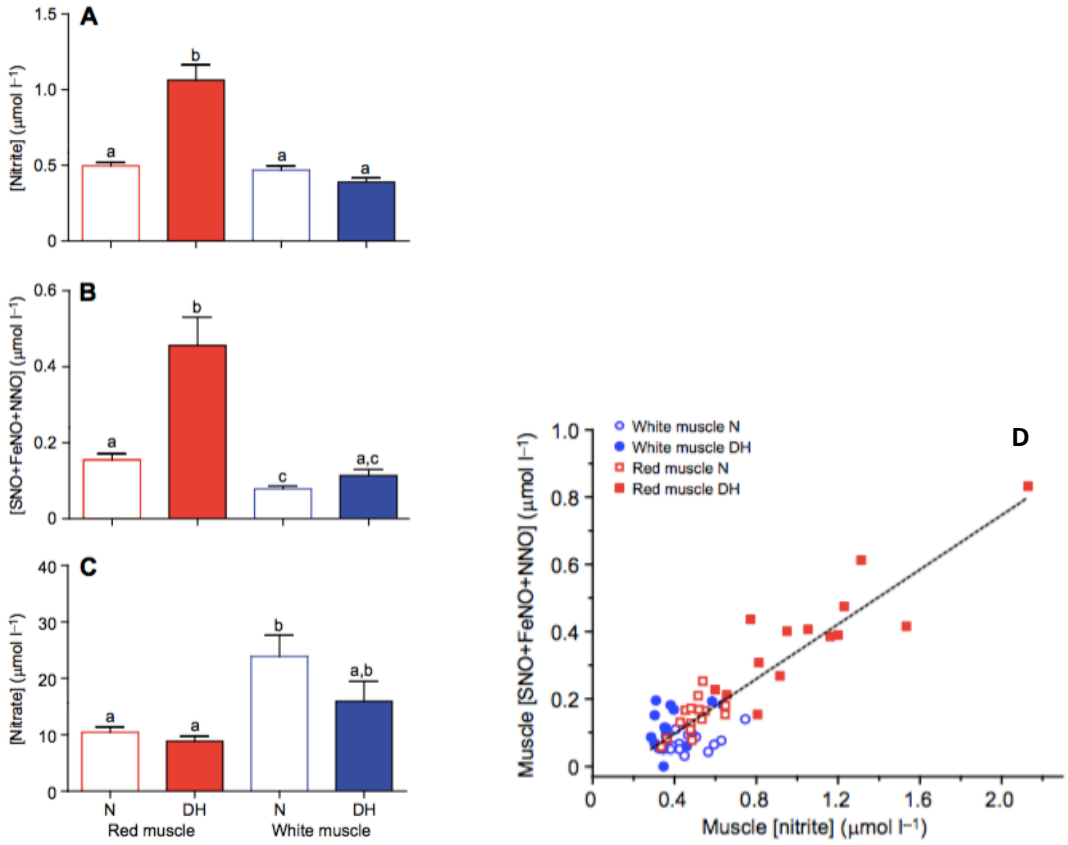

Fig. 11. Red and white muscle NO metabolites in normoxic and deeply hypoxic crucian carp. Concentrations of nitrite (A), SNO+FeNO+NNO(B) and nitrate (C) in red muscle and white muscle of crucian carp exposed to normoxic (N; PO2>140 mmHg) and deeply hypoxic (DH; 1<PO2<3 mmHg) water for 1 day. Values are means±s.e.m. (N=15 for each group). (D) Relationship between SNO+FeNO+NNO and nitrite concentration in muscle tissues from normoxic and deeply hypoxic crucian carp.

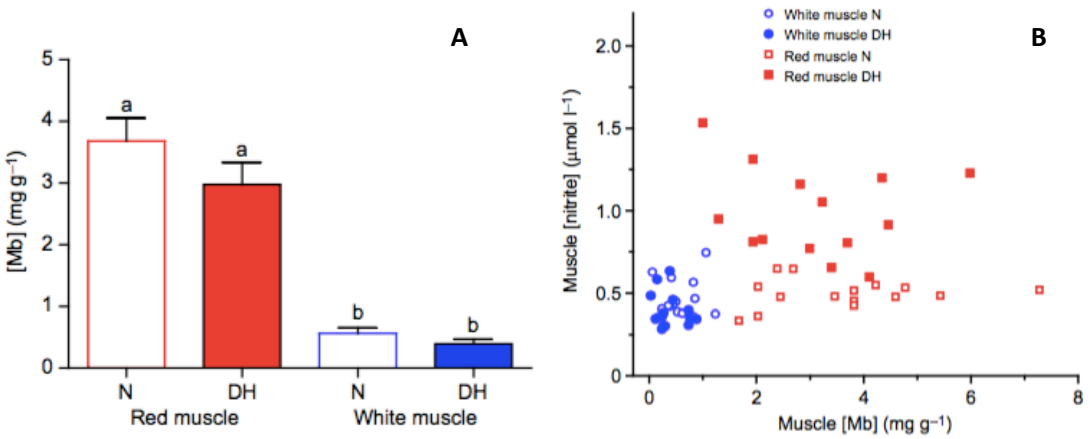

Fig. 12. Mb concentration in red and white muscle from normoxic and deeply hypoxic crucian carp.
(A) Mb concentration is given in mg per g wet mass for crucian carp exposed to normoxia (N; PO_2>140 mmHg) and deep hypoxia (DH; 1<PO_2<3 mmHg) for 1 day. Values are means±s.e.m. (N=15 for each group). (B) Red (red symbols) and white (blue symbols) muscle nitrite levels as a function of Mb concentration in crucian carp exposed to normoxia and deep hypoxia (N=60 in total).

2.3.2. Nitrate reductase activity

In white skeletal muscle homogenates from both normoxic and deeply hypoxic fish, there was no significant nitrite production during 5 h hypoxic incubation in the absence of exogenous nitrate (Fig. 13A,E and C,G). However, when 300 µmolL^{-1} nitrate was added, the muscle tissue from deeply hypoxic fish showed a significant nitrite production (Fig. 13D,H). In muscle from normoxic fish, the change in nitrite concentration was not significant (P=0.07) (Fig. 13B,F). In the liver, we obtained similar results from N_2 incubations in Eppendorf tubes and in rotating tonometers with continuous flow of N_2 (Fig. 14). The nitrite production from nitrate in liver was significant after 5 h incubation in the presence of added nitrate (Fig. 14), and the nitrate reductase activity was higher than that in muscle. For comparison, the nitrite production in a single mouse liver was tested and it was found a 3 times higher than in crucian carp (data not shown). The nitrite production in liver homogenates from crucian carp was significantly inhibited by allopurinol (Fig. 14 I). No nitrate reduction was observed in the heart, and the nitrite concentration actually decreased by about 2 µmolL^{-1} during 5 h of incubation (Fig. 15).

2.3.3. Ethanol production

Ethanol was not detected in white muscle and plasma from normoxic fish, whereas ethanol increased to 10.5±1.1 mmol L^{-1} (mean±s.e.m., N=8) in muscle and 3.9±0.3 mmol L^{-1} (mean±s.e.m., N=8) in plasma of crucian carp exposed to deep hypoxia for 1 day.

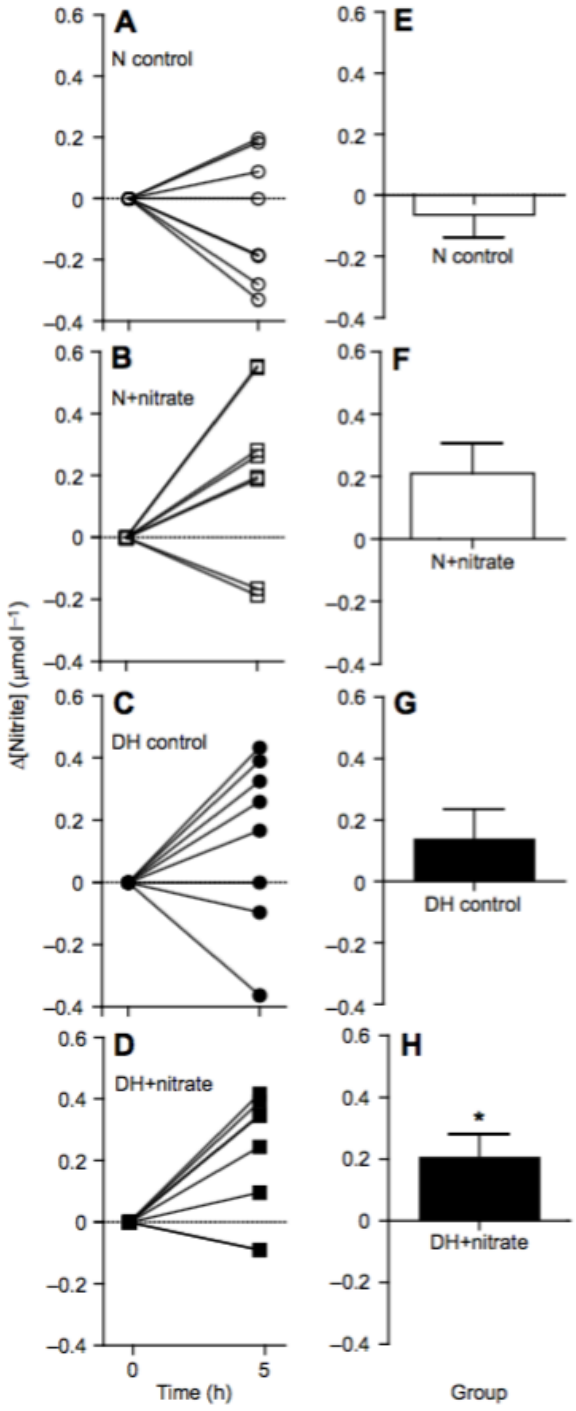

Fig. 13. Nitrate reductase activity in white muscle homogenates.
Muscle homogenates are from normoxic (N) and deeply hypoxic (DH) crucian carp. (A–D) Change in nitrite concentration in individual samples during 5 h of N2 incubation with (squares) or without (circles) 300 μmol l^{-1} exogenous nitrate added to the homogenates. (E–H) Group-specific changes as means±s.e.m.

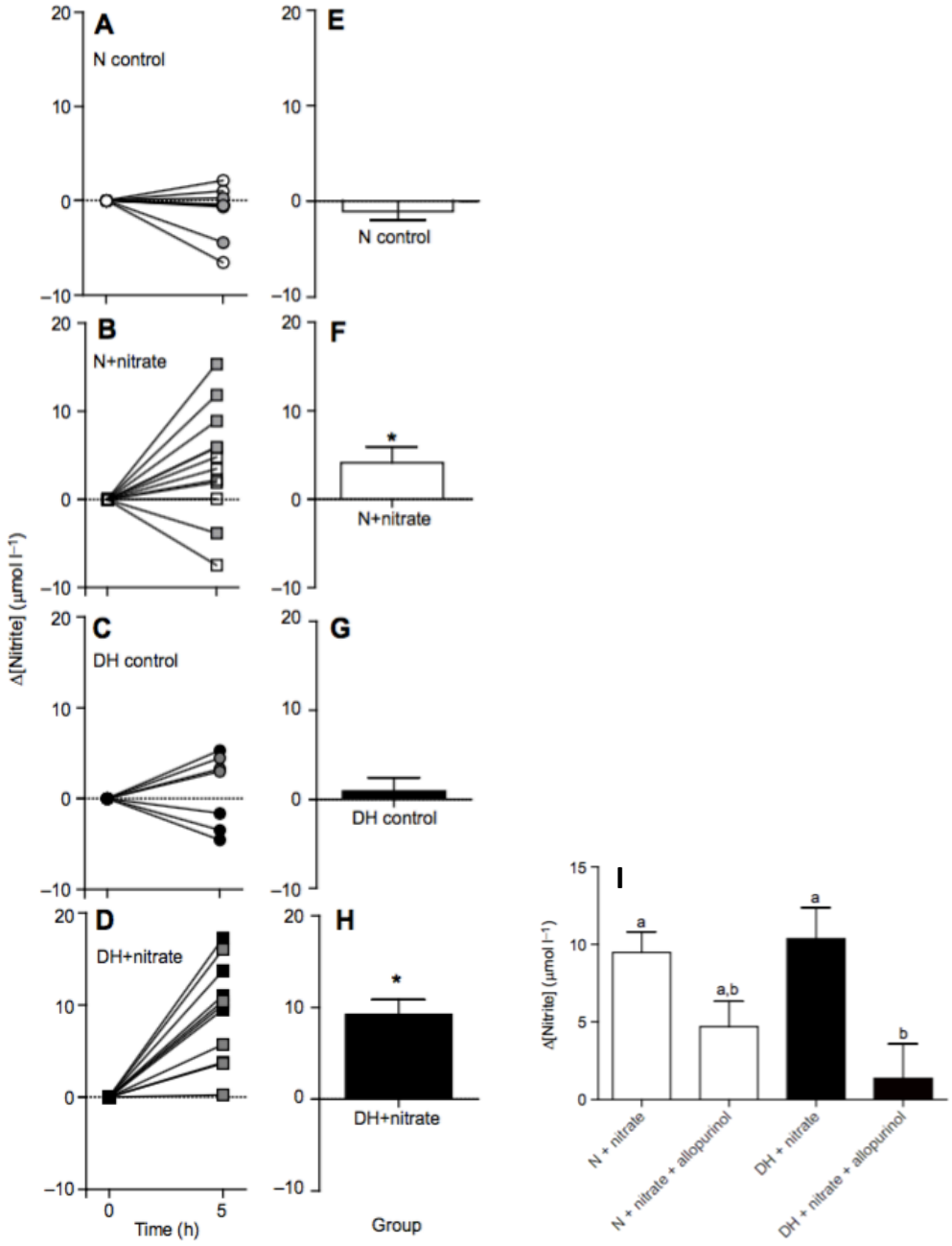

Fig. 14. Nitrate reductase activity in liver homogenates.
Liver homogenates are from normoxic (N) and deeply hypoxic (DH) crucian carp. (A–D) Change in nitrite concentration in individual samples during 5 h of N_2 incubation with (squares) or without (circles) 300 μmol L^{-1} exogenous nitrate added to the homogenates. Black and white symbols are from incubations in Eppendorf tubes and gray symbols signify incubation in tonometers (E–H) Group-specific changes as means±s.e.m. (I) Effect of allopurinol on nitrate reductase activity in liver homogenates. All groups (means±s.e.m.) were incubated with 300 μmolL^{-1} nitrate for 5 h with or without 2 mmolL^{-1} allopurinol (N=5 in each group).

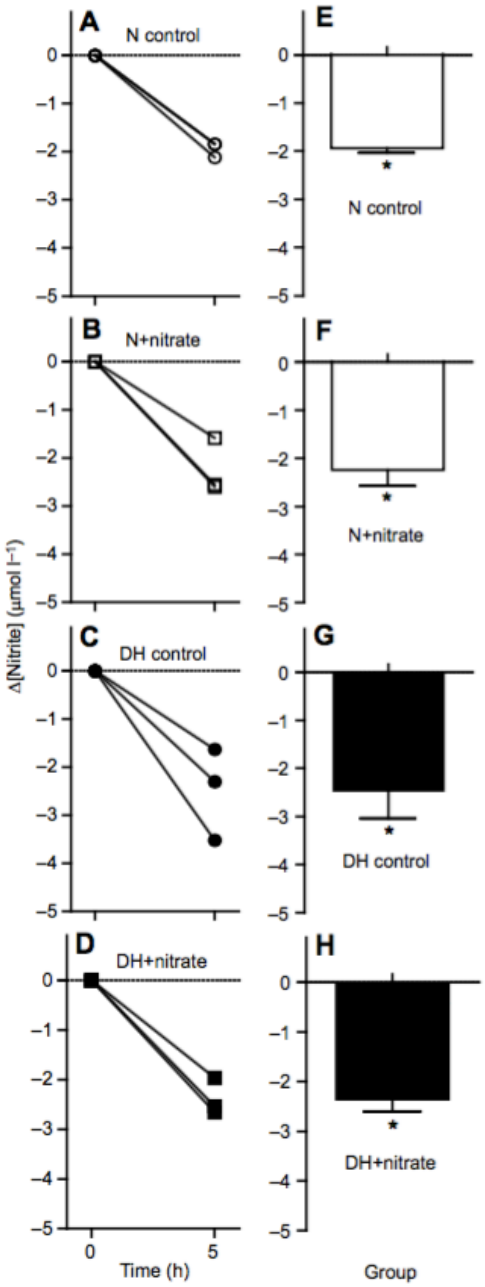

Fig. 15. Test for nitrate reductase activity in heart tissue.
Ventricle homogenates are from normoxic (N) and deeply hypoxic (DH) crucian carp. (A–D) Change in nitrite concentration in individual samples during 5 h of N_2 incubation with (squares) or without (circles) 300 μmol L^{-1} exogenous nitrate added to the homogenates. (E–H) Group-specific changes as means±s.e.m. A one-sample t-test was performed to compare group means with zero; asterisks signify statistical difference ($P<0.05$). Because of the small ventricle size, ventricles from two fish were pooled in each individual homogenate (N=3 for each group).

2.4. DISCUSSION

The present study examined NO metabolites and Mb levels in both red and white muscle in a hypoxia-tolerant lower vertebrate. Mb concentrations were considerably higher in red muscle than in white muscle, and the level of nitrite increased significantly with exposure to deep hypoxia in red muscle, but not in white muscle. The latter observation corroborates the idea that tissues rich in Mb and mitochondria, such as the red muscles and the heart, develop increased nitrite levels during severe O_2 deprivation (Jensen et al., 2014).

Hypoxia or anoxia has previously been found to increase nitrite concentration in the heart of crucian carp exposed to 1–5 days of full anoxia (Sandvik et al., 2012) or after 1 day to deep hypoxia ($1<PO_2<3$ mmHg) suggesting that deep hypoxia (rather than full anoxia) is sufficient to stimulate an elevation of cardiac nitrite concentration (Hansen et al., 2016). Increases in nitrite concentration in response to hypoxia or anoxia have been also reported in pectoral muscle of slider turtles, having a high proportion of red muscle fibers. On the contrary, no changes in nitrite concentration were observed in the white muscle of crucian carp and goldfish exposed to prolonged hypoxia or anoxia (Hansen and Jensen, 2010; Sandvik et al., 2012; Jensen et al., 2014).

In this study, the hypoxia-induced increase in red muscle nitrite concentration was mirrored by an increase in nitros(yl)ation products (Fig. 11) and a strong linear correlation between muscle nitrite concentration and [SNO+FeNO+NNO] was found to apply to both normoxic and deeply hypoxic red and white myocytes (Fig. 11D). This substantiates the nitrosative power of nitrite, where nitrite (via nitrosating species like N_2O_3) generates SNO compounds, and supports that NO originating from nitrite reduction nitrosylates heme groups to form FeNO compounds. The overall response of increased nitrite and nitros(yl)ation compounds in red muscle during deep hypoxia resembles the increased nitrite/NO activity found in cardiomyocytes from deeply hypoxic and anoxic crucian carp and slider turtles (Sandvik et al., 2012; Jensen et al., 2014; Hansen et al., 2016).

It has been hypothesized that Mb may be involved in maintaining/increasing intracellular nitrite levels in hypoxia-tolerant species (Jensen et al., 2014), and the

present study therefore tested for a correlation between muscle nitrite and Mb levels. The levels of Mb in red muscle and white muscle of normoxic crucian carp (Fig. 12B) are similar to those reported in other fish species (Reynafarje, 1963; Giovane et al., 1980; Jaspers et al., 2014). Additionally, 1 day of deep hypoxia did not change the Mb protein level, which is in accordance with other studies on Mb expression during hypoxia, where 2–5 days of hypoxia in killifish, goldfish and carp did not affect Mb concentration in heart or red muscle (Cossins et al., 2009; Borowiec et al., 2015) or Mb mRNA levels (Roesner et al., 2008; Okogwu et al., 2014). If Mb concentration had a direct influence on muscle nitrite levels, one would expect a positive correlation between muscle nitrite and muscle Mb concentration. However reported data reflect an increased red muscle nitrite concentration that is independent of muscle Mb concentration (Fig. 12B).

Other possible mechanisms for elevating intracellular nitrite levels should also be considered. As high mitochondrial content is also a shared property of red muscle and cardiomyocytes, mitochondria could be involved in elevating intracellular nitrite concentration during deep hypoxia. Sequestering of nitrite inside mitochondria would be much in line with the fact that the cytoprotective effects of nitrite are largely directed at the mitochondria (Halestrap, 2004; Walters et al., 2012; de Lima Portella et al., 2015). In the mitochondria, nitrite S-nitrosates complex I, attenuating ROS generation during early reperfusion (Dezfulian et al., 2009; Chouchani et al., 2013), and nitrosylates complex IV, which inhibits oxygen consumption rates (Hendgen-Cotta et al., 2008). However, a role of nitrite binding/storage by mitochondria is not known and future studies are needed to clarify the possible role of mitochondria in elevating cellular nitrite levels during hypoxia and anoxia.

This study showed that the crucian carp possess a tissue nitrate reductase activity able to reduce nitrate to nitrite. The nitrate reduction activity was higher in liver (Fig. 14) than in muscle (Fig. 13) and it was absent in the heart (Fig. 15). In the heart, the nitrite concentration actually decreased, pointing at a dominating nitrite reduction under the assay conditions. Nitrate reductase activity in red muscle was not tested because the limited amount tissue. However, given the low nitrate reduction activity in white muscle and its absence in the heart (resembling red muscle metabolically),

the nitrate reduction activity may also be low in red muscle, which is supported by the absence of a decrease in red muscle nitrate concentration in deeply hypoxic fish (Fig. 11C). The higher nitrate reduction in the liver will supply nitrite to the anoxic liver, but it may additionally function to export nitrite to other tissues, including the heart via the circulation, along with the function of the liver to deliver glucose to sustain glycolytic metabolic activity during deep hypoxia.

The difference in nitrate reductase activity between tissues corresponds to findings in mice and rats, where liver nitrate reductase activity is high compared with that in heart and muscle (Jansson et al., 2008; Piknova et al., 2015). Interestingly, in the current study, the nitrate reductase activity appeared higher in tissues from crucian carp exposed to deep hypoxia for 1 day than in tissues obtained from normoxic fish (Fig. 13 D,H, and 14 D,H), which suggests that the enzyme(s) responsible for nitrate reduction in crucian carp may be upregulated with transition to deep hypoxia. One main enzyme responsible for nitrate reduction is XOR, as reflected by a significant (but incomplete) inhibition of nitrate reductase activity using the XOR inhibitor allopurinol (Fig. 14I). This is similar to mammals (Jansson et al., 2008; Piknova et al., 2015), where a role for XOR is further substantiated by the finding of high nitrate reductase activity in the liver and gastrointestinal tract (Jansson et al., 2008), which have the highest XOR levels (Harrison, 2004). In mammals, XOR expression is upregulated by hypoxia (Kelley et al., 2006), and also in germ-free mice, where nitrate reduction by commensal bacteria is absent (Huang et al., 2010). XOR is known to also be expressed in teleost fish (Basha and Rani, 2003; Garofalo et al., 2015). Thus, XOR is a likely candidate for nitrate reductase activity in crucian carp, and given that the expression pattern is similar to that in mammals, this would explain the difference between nitrate reductase activity in crucian carp liver and muscle. An upregulation of XOR (and/or another nitrate reductase enzyme) in crucian carp would explain the increase in tissue nitrate reductase activity in tissues from deeply hypoxic fish compared with normoxic fish. It is also possible that other nitrate reductase enzymes, expressed solely during hypoxia, contribute to nitrate reduction in crucian carp. This awaits future study.

2.5. CONCLUSIONS CHAPTER 2

In summary, this study supports the role of nitrite as alternative pathway for NO generation during hypoxia exposure. Results showed that during deep hypoxia, nitrite levels increase in tissues major affected by oxygen depression, such as the heart and the red musculature of crucian carp. This is not directly paralleled by high Mb levels; future studies will determine whether this increase in intracellular nitrite may relate to the high mitochondria content that characterizes cardiomyocytes and red muscle fibers. Moreover, this study provides evidence of a low nitrate reductase activity in crucian carp tissues that can supplement other nitrite supply routes and probably contributes to cytoprotection under conditions of deep hypoxia.

GENERAL CONCLUSIONS

The results reported in this Ph.D. thesis strongly suggest that, if exposed to humoral and environmental influences, the fish heart undergoes significant molecular, structural, and physiological remodelling. Fish heart flexibility has been here demonstrated in response to both AngII treatment and low oxygen availability. Chapter 1 shows as chronic exposure to AngII elicits cardiac morpho-functional readjustments in the eel *A. anguilla* and in the zebrafish *D. rerio.* In the eel, this occurs through molecular mechanisms which involve modification of NO production and changes in the expression and localization of molecules which regulate NOS activity.
Chapter 2 provides evidence on physiological and molecular mechanisms that, in the crucian carp exposed to deep hypoxia, lead to an increase in cardiac nitrite concentration; this, stabilizing NO levels, allows cardioprotection.
Although much still remains to be investigated, the results here reported clearly indicate the central role played by the cardiac NOS/NO system as a major coordinator/integrator of molecular signal cascades which control cardiac adaptive responses in fish. Overall, this information suggest that extrinsic (humoral agents) and environmental (hypoxia) factors can fine-regulate fish heart form and function by converging on common molecular networks that operate for connecting and integrating information.
Future research will contribute to better decipher the complex molecular networks involved in the cardiac plasticity in fish. In this context, the choice of appropriate animal models, characterized by a remarkable morpho-functional adaptability to a variety of environmental factors (temperature, partial pressure of oxygen, pH, environmental pollutants, etc.) could be of help.
In an evolutionary and comparative perspective, we hope that the information on the molecular strategies of cardiac regulation in fish will cross the boundaries of comparative, integrative and environmental physiology, emerging also as a topic of interest and a source of bioinspiration for human cardiovascular physiology.

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Angiotensin II dependent cardiac remodeling in the eel *Anguilla anguilla* involves the NOS/NO system

Mariacristina Filice [a, 1], Daniela Amelio [a, 1], Filippo Garofalo [a, 1], Sabrina David [b], Alberto Fucarino [b, c], Frank Bo Jensen [d], Sandra Imbrogno, PhD [a, *], Maria Carmela Cerra, PhD [a, **]

[a] Department of Biology, Ecology and Earth Sciences (B.E.ST), University of Calabria, Arcavacata di Rende, CS, Italy
[b] Department of Experimental Biomedicine and Clinical Neuroscience, University of Palermo, Palermo, Italy
[c] Euro-Mediterranean Institute of Science and Technology, Palermo, Italy
[d] Department of Biology, University of Southern Denmark, Campusvej 55, DK-5230 Odense, Denmark

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ABSTRACT

Angiotensin II (AngII), the principal effector of the Renin-Angiotensin System (RAS), plays an important role in controlling mammalian cardiac morpho-functional remodelling. In the eel *Anguilla anguilla*, one month administration of AngII improves cardiac performance and influences the expression and localization of molecules which regulate cell growth.

To deeper investigate the morpho-functional chronic influences of AngII on the eel heart and the molecular mechanisms involved, freshwater eels (*A. anguilla*) were intraperitoneally injected for 2 months with AngII (1 nmol g BW^{-1}). Then the isolated hearts were subjected to morphological and western blotting analyses, and nitrite measurements.

If compared to control animals, the ventricle of AngII-treated hearts showed an increase in *compacta* thickness, vascularization, muscle mass and fibrosis. Structural changes were paralleled by a higher expression of AT_2 receptor and a negative modulation of the ERK_{1-2} pathway, together with a decrease in nitrite concentration, indicative of a reduced Nitric Oxide Synthase (NOS)-dependent NO production. Moreover, immunolocalization revealed, particularly on the endocardial endothelium (EE) of AngII-treated hearts, a significant reduction of phosphorylated NOS detected by peNOS antibody accompanied by an increased expression of the eNOS disabling protein NOSTRIN, and a decreased expression of the positive regulators of NOS activity, pAkt and Hsp90. On the whole, results suggest that, in the eel, AngII modulates cardiac morpho-functional plasticity by influencing the molecular mechanisms that control NOS activity and the ERK_{1-2} pathway.

1. Introduction

Angiotensin II (AngII), the active end-product of the Renin-Angiotensin System (RAS), is an octapeptide hormone that plays important roles in the regulation of cardiovascular homeostasis and fluid osmolarity.

In mammals, AngII interacts with plasma membrane receptors named AT_1 and AT_2 (for references see Ref. [1]) to modulate various aspects of cardiac function, including chronotropism, inotropism, and coronary vasomotility [2]. Moreover, by cross-talking with growth-promoting and growth-inhibiting factors, as well as with angiogenic molecules, AngII induces mammalian cardiac growth and remodelling, particularly under pathologic conditions [3,4]. Within the complex humoral balance that controls mammalian

Abbreviations: ACE, Angiotensin Converting Enzyme; Akt, protein kinase B; AngII, Angiotensin II; cGMP, cyclic Guanosine Mono-Phosphate; EE, Endocardial Endothelium; eNOS, endothelial Nitric Oxide Synthase; ERK1-2, Extracellular Signal-Regulated Kinases1-2; Hsp-90, Heat shock protein 90; NO, Nitric Oxide; NOS, Nitric Oxide Synthase; NOSTRIN, eNOS trafficking inducer; PBS, phosphate-buffered saline; PKG, protein kinase G; RAS, Renin-Angiotensin-System.

* Corresponding author. Lab. of Organ and System Physiology, Dept of Biology, Ecology and Earth Sciences, University of Calabria, 87036 Arcavacata di Rende, CS, Italy.

** Corresponding author. Lab. of Organ and System Physiology, Dept of Biology, Ecology and Earth Sciences, University of Calabria, 87036 Arcavacata di Rende, CS, Italy.

E-mail addresses: sandra.imbrogno@unical.it (S. Imbrogno), maria_carmela.cerra@unical.it (M.C. Cerra).

[1] These authors equally contributed to the work.

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cardiac form and function, AngII interacts with the Nitric Oxide Synthase (NOS)/Nitric Oxide (NO) system. By recruiting AT_1 and/or AT_2-mediated transduction pathways, AngII decreases NO bioavailability; in turn, by down-regulating the synthesis of Angiotensin Converting Enzyme (ACE) and AT_1 receptor, NO counteracts AngII-mediated actions [5,6].

A functional RAS, homologus to that found in mammals, is present also in fish (see Ref. [7]). In teleost, AngII was identified and sequenced in various species, including the chum salmon *Oncorhynchus keta* [8], the Japanese goosefish *Lophius litulon* [9] and the American eel *Anguilla rostrata* [10]. In parallel, several studies provided a molecular and biochemical characterization of piscine AngII receptors (see for example [11,12]). A cDNA sequence (GenBank accession number AJ005132 [12]), with 60% homology with mammalian AT_1, was reported in the European eel *A. anguilla* [13]. A receptor sharing the origin with mammalian AT_2 has been observed in the Japanese eel *A. japonica* [14]; lastly, an AT receptor able to bind mammalian anti-AT_2 antibody was detected in the cardiac tissues of *A. anguilla* [15].

The teleost heart is sensitive to both the short- and medium/long-term modulation exerted by AngII (see for references [2,13,15–17]). Among teleost, the eel represents an experimental model till now largely used to decipher the complex modulatory neuro-humoral networks involved in the fish heart modulation, particularly in relation to the role of NO as a major organizer of basal cardiac physiology and a key coordinator of physically and chemically activated intracellular signalling (see Ref. [18] for references). This is due to its great flexibility in relation not only to the basic cardiac design and elaborated neuroendocrine traits, but also to its biochemical-metabolic plasticity and acclimatory potentialities (see Ref. [19] for references). Moreover, its relatively simple cardiac design allows setting up *in vitro* working heart preparation which, mimicking the haemodynamic response of the *in vivo* heart, generates physiological values of cardiac parameters [20].

By using an isolated and perfused working heart, it has been demonstrated that in the eel *A. anguilla* AngII exerts a direct mechanical cardio-suppression mediated by a G protein-coupled AT_1-like receptor that, in turn, triggers a NO-cGMP-Protein Kinase G (PKG) signal transduction pathway [2]. Moreover, if administered for a month, AngII improves the performance of the eel heart, enhancing its ability to sustain increased afterload [15]. These effects involve the AT_2 receptor, and factors which regulate cell growth [i.e. c-kit, heat shock protein 90 (Hsp-90), NO] and apoptosis [i.e. apoptosis repressor with CARD domain (ARC)].

Based on these premises, the aim of this study was to analyze morpho-functional influences exerted on the eel (*A. anguilla*) heart by chronic (two months) exposure to AngII. In view of the crucial role of NO in the mechanisms of cardiac rearrangement [21,22], the interplay between AngII and the NOS/NO system was also analysed.

The results evidenced an influence of AngII on the structural remodeling of the eel heart. This is suggested by an increase in the *compacta* thickness, ventricle muscle mass, vascularisation, and in the amount of collagen fibres, both at perivascular and interstitial level, observed in AngII-treated hearts. Structural modifications were paralleled by a higher expression of the AT_2 receptor, a negative modulation of the phosphorylated extracellular signal-regulated $kinases_{1\text{-}2}$ ($ERK_{1\text{-}2}$), and by changes in the expression and localization of molecules which regulate NOS activity, such as the protein kinase B (Akt), the eNOS disabling protein NOSTRIN, and the heat shock protein 90 (Hsp90).

2. Materials & methods

2.1. Animals

Specimens of freshwater European eel (*A. anguilla*) weighing 83,31 ± 5,39 g (mean ± SD; n = 27), provided by a local hatchery, were kept in aerated freshwater at room temperature (18–20 °C) for 8 weeks and fed twice a week with commercial fish food. Each eel was anesthetized with tricaine methanesulfonate (MS222, SIGMA-Aldrich Chemical Co., UK). Animal care and procedures were in accordance with the U.S. National Institutes of Health's Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1996) and with Directive 2010/63/EU.

Two groups of animals have been used. Through intraperitoneal injections on alternate days, each animal has been treated, for 8 weeks, with:

- Group 1 (n = 13): 1 ml of physiological saline.
- Group 2 (n = 14): 1 nmol AngII gBW^{-1} in 1 ml of physiological saline.

Animal treatment with saline or AngII and heart samples collection were performed during 2013, i.e. before the new Italian law about the care and use of Laboratory Animals, effective from March 2014, in which the European eel is considered an endangered species.

2.2. Chemicals

The homologue teleost octapeptide AngII [17] was purchased from SIGMA. Solution was prepared in double-distilled water; dilutions were made in physiological saline immediately before use.

2.3. Experimental protocols

2.3.1. Morphological analysis

Hearts from control and AngII treated eels (n = 3 for each condition) were fixed in MAW (methanol:acetone:water, 2:2:1) for light microscopy and immunofluorescence (for details see Ref. [15]).

2.3.1.1. Collagen staining. Sections were stained with Sirius red for collagen fibers detection. Slides were observed under a light microscope (Zeiss Axioscope); images were digitalized by Axiocam 105 color (Zeiss).

2.3.1.2. Semithin sections. Hearts from control and treated eels (n = 3 for each condition) were fixed in 2.5% glutaraldehyde. Small cubes of tissue were taken from the middle anterior wall of the hearts. The pieces were dehydrated, embedded in EPON 812 (Shell Chemical Co., San Francisco, CA) and cutted (0.70 μm) with an ultramicrotome (Ultra Cut, Leica). Sections were stained with methylene blue, and observed with LEICA optical microscope (LEICA DM5000B). Images were digitalized by using Adobe Photoshop 7.0 and the morphometrical evaluations (*compacta* thickness, percentage of muscular and vascular compartment) were carried out on images (10X magnification) using ImageJ 1.49v. Geometrical scaling was performed prior to start measurements.

Compacta thickness was quantified by measuring the distance, from the border, between the epicardium and endocardium. The percentage of surface area occupied by empty spaces was calculated by thresholding on random images of different transverse and longitudinal ventricular sections. The resulting area (in pixels) was subtracted from the total area (in pixels) of the section, thus obtaining the overall area of myocardium and vascular components. The myocardial surface was obtained by subtracting from the overall area (myocardium plus vascular components) the vascularized area (percentage of area occupied by vessels), quantified by measuring each blood vessel (Adobe Photoshop CC15.2Portable).

2.3.1.3. Immunofluorescence. Sections were rinsed in TBS and incubated with 1.5% BSA in TBS for 1 h. They were then incubated overnight at 4 °C with rabbit polyclonal antibodies directed against NOSTRIN (cat# Sc-134803), AT_2 receptor (cat# Sc-48452), and goat polyclonal anti pNOS3-Ser1177 (cat# Sc-12972), diluted 1:100 in TBS. All antibodies were from Santa Cruz Biotechnology, Inc., Heidelberg, German. For signal detection, after washing in TBS (3 × 10 min), slides were incubated with FITC-conjugated anti rabbit and anti-goat IgG (SIGMA, 1:100) and mounted with mounting medium (Vectashield, Vector Laboratories Burlingame, CA, USA). Slides were observed under a fluorescence microscope (Axioscope, Zeiss), and the images were digitalized by Axiocam 105 color (Zeiss). Negative controls were obtained on parallel sections treated in the same manner, excluding primary antibody. For nuclear counterstaining, sections were incubated with Propidium iodide (SIGMA; 1:10,000) for 5 min.

2.3.2. Nitrite measurement

For nitrite measurements, hearts from control and AngII-treated animals (n = 4 for each condition) were washed in a phosphate-buffered saline [50 mM phosphate buffer; pH 7.8; 85 mM NaCl; 2.4 mM KCl; 10 mM N-ethylmaleimide (NEM); 0.1 mM diethylenetriaminepentaacetic acid (DTPA)], and then dried on a paper towel, weighed and frozen in liquid N_2. Each heart was homogenized in 50 mM phosphate buffer (4 μL mg^{-1} tissue; pH 7.3), containing 10 mM NEM (N-ethylmaleimide) and 0.1 mM DTPA (diethyletriaminepentaacetic acid) to stabilize S-nitrosothiols. Samples were centrifuged (2 min, 16,000 g, 4 °C) and the supernatant was immediately measured. Nitrite was measured by reductive chemiluminescence, using a Sievers (Boulder, CO, USA) NO Analyzer (NOA, model 280i) and previously described procedures (see Ref. [23] for references).

2.3.3. Western blotting and densitometric analysis

To evaluate whether chronic AngII treatment affects the expression pattern of protein involved in cellular growth and NO metabolism, western blotting analysis was performed on heart extracts. Eel hearts of group 1 (control) and 2 (AngII treated) (n = 3 for each condition) were rapidly immersed in liquid nitrogen and stored at −80 °C. The ventricle, separated from the atrium and the bulbus arteriosus, was prepared according to Amelio et al. [24]. Amounts of 60 μg of proteins were separated on 8% SDS-PAGE gel (for eNOS/peNOS detection) or 12% SDS-PAGE gels (for $ERK_{1\text{-}2}$/$pERK_{1\text{-}2}$, NOSTRIN, Hsp90, Akt/pAkt, AT_1 and AT_2), and electroblotted on to a nitrocellulose membrane. For immunodetection, blots were incubated overnight at 4 °C with either rabbit polyclonal antibodies directed against NOSTRIN, Akt1/2/3 (cat# Sc-8312), pAkt1/2/3-Ser473 (cat# Sc-7985-R), ERK_2 (cat# Sc-154), eNOS (cat# N3893), or mouse monoclonal antibodies directed against AT_1 receptor (cat# Sc-57036), $pERK_{1\text{-}2}$ (cat# Sc-7383), or goat polyclonal antibody directed against pNOS3-Ser1177, Hsp90 (cat# Sc-1055), AT_2 receptor. eNOS was purchased from SIGMA; all other antibodies were from Santa Cruz Biotecnology.

Peroxidase linked secondary antibodies (anti-rabbit and anti-goat) (Amersham) were diluted 1:2000 in TBS-T containing 5% non-fat dry milk. Immunodetection was performed by using an enhanced chemiluminescence kit (ECL PLUS, Amersham). Autoradiographs were obtained by exposure to X-ray films (Hyperfilm ECL, Amersham). Immunoblots were digitalized and the densitometric analysis of the bands was carried out using WCIF Image J based on 256 grey values (0 = white; 256 = black). Quantification of the bands was obtained by measuring (eight times on each band) the mean optical density of a square area, after the background has been subtracted.

2.3.4. Statistics

Compacta thickness and vascularization rate were calculated on 5 images for each group; values represent the means ± SEM of 6 measurements for each image. Statistical significance of differences was assessed using the Student's *t-test* ($^*p < 0.05$; $^{**}p < 0.005$).

For densitometric analyses, values were expressed as means ± SEM of absolute values from individual experiments; statistic was assessed by unpaired *t-test* ($^{**}p < 0.005$; $^{***}p < 0.0005$). GraphPad Prism software, version 4 (GraphPad Software, San Diego, CA) was used for all the statistical analysis.

3. Results

3.1. Cardiac morphometry

Ventricular sections stained with Sirius red (Fig. 1A and B) showed collagen fibers localized both in the *compacta* and *spongiosa*. A higher amount of collagen fibers, located around subepicardial vessels, in coronary vessels wall, at the border between *compacta* and *spongiosa,* and in the interstitium, has been detected in the *compacta* with respect to the *spongiosa*. In the *spongiosa*, collagen fibers localized in the subendocardium and within interstitial spaces. The hearts of AngII-treated animals show an increase of collagen amount in all the above described structures (Fig. 1B).

Observations carried out on semithin sections (Fig. 1C and D) show a significant increase of ventricle muscularity in treated eels, associated with an increased thickness of the *compacta* and trabecular size in the *spongiosa*. AngII-treated animals also show an increased *compacta* vascular compartment (Fig. 1E, G, F).

3.2. AngII receptors

To evaluate the influence of chronic AngII exposition on the cardiac expression of AT_1 and AT_2 receptors, western blotting analysis and immunofluorescence were performed by using mammalian anti-AT_1 and AT_2 antibodies.

AT_1 receptor was undetectable by both immunoblotting and immunofluorescence. In contrast, densitometric analysis of the blot with anti-AT_2 antibody revealed an increased expression of this receptor in AngII-treated hearts, with respect to the control (Fig. 2D). Immunofluorescence showed a different localization of AT_2 in the hearts of control and treated animals. In particular, AT_2 signal was revealed in the myocardiocytes of control hearts (Fig. 2A), and at the EE of AngII-treated hearts (Fig. 2B).

3.3. $ERK_{1\text{-}2}$

As revealed by western blotting analysis performed on cardiac extracts, prolonged exposition to AngII elicits a significant decrease of $ERK_{1\text{-}2}$ phosphorylation in the hearts of AngII-treated animals (Fig. 3A). This implicates a negative modulation of the $ERK_{1\text{-}2}$ signaling in the chronic effects of AngII in the eel heart.

3.4. NOS/NO system

To evaluate the influence of AngII treatment on the cardiac NO production, the concentration of nitrite was determined in heart homogenates of both control and AngII-treated eels. Results showed a significant decrease of nitrite concentration in hearts from treated animals compared with the control group (control: 1.33 ± 0.13 μmol L^{-1}; AngII-treated: 0.60 ± 0.04 μmol L^{-1}).

Western blotting of ventricular extracts revealed no significant differences in eNOS phosphorylation between control and treated hearts (data not shown) accompanied by a slight decrease of pAkt expression in treated animals (Fig. 3B). Moreover,

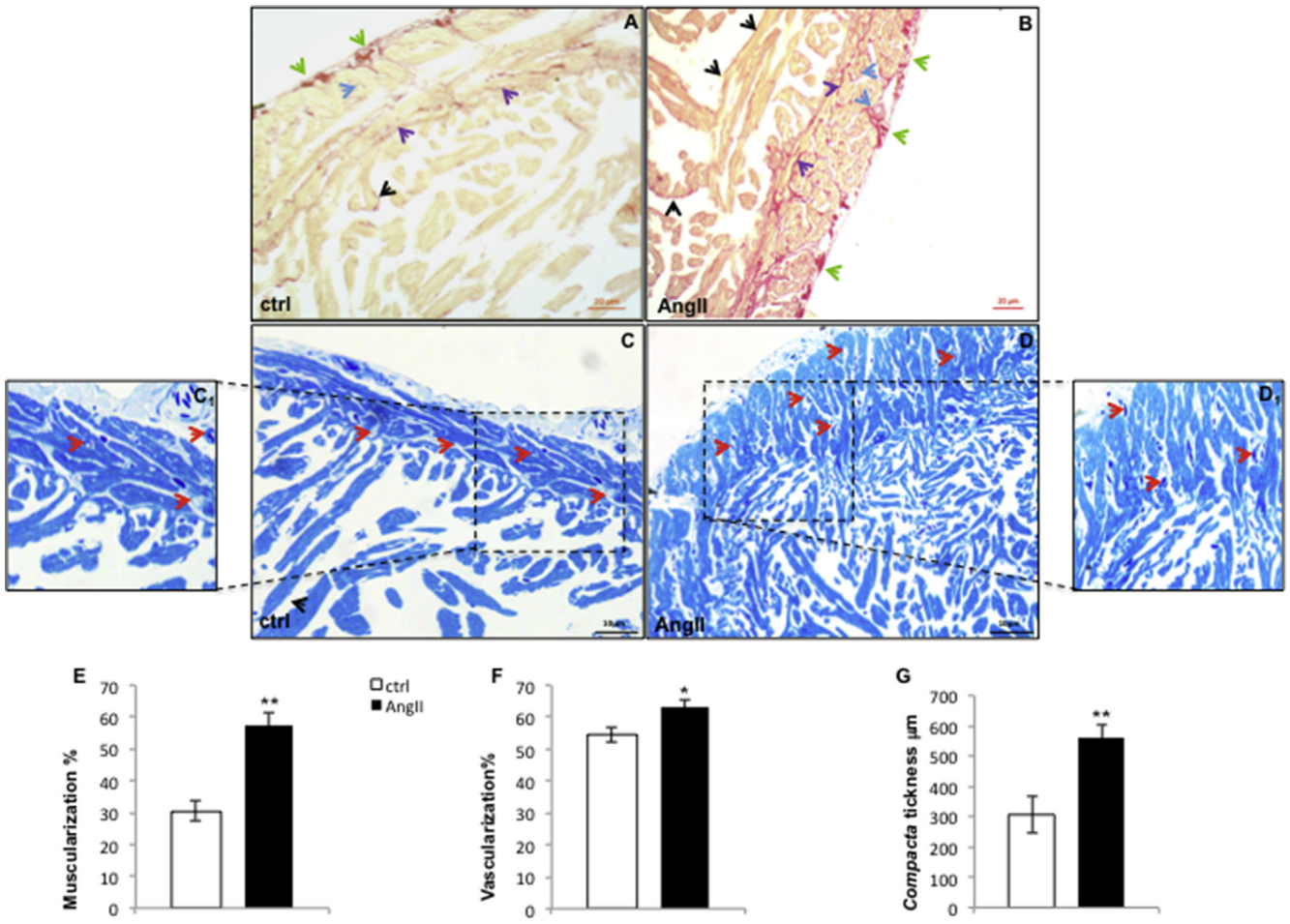

Fig. 1. Representative images showing morphological changes occurring in the eel ventricle after AngII treatment. (A, B) Sirius red staining of control (A) and AngII (B) treated hearts. Collagen fibers localize at subepicardial level (green arrows), in the vessels wall (blue arrows), at subendocardial level (black arrows) and in the interstitium (violet arrows). (C, D) Methylene Blue stained ventricular cardiac semithin sections of control (C) and AngII (D) treated eels. After treatment, the increment of *compacta* thickness, and of muscular and vascular (red arrows) components is evident. Inset C_1 and D_1 represent higher magnification of the vascularized area of control and AngII treated hearts, respectively. Statistical significances are reported in the corresponding histograms (E, F, G) (*$p < 0.05$; **$p < 0.005$). Values represent the means ± SEM of 6 measurements for each image ($N = 5$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

immunofluorescence showed a different localization of peNOS in the heart of control and treated animals. In particular, while in the control hearts, a peNOS signal was captured at the EE level and in the cardiomyocytes of ventricle (Fig. 4A) and atrium (Fig. 4B), in treated hearts the enzyme prevalently localized in the myocardiocytes (Fig. 4D and E), only a weak signal appearing on the EE. In addition, at vascular level, a reduction of the peNOS signal (Fig. 4G) was observed in the vascular endothelium of treated animals, with respect to the control (Fig. 4F).

In line with the reduction of pAkt expression, western blotting analysis revealed a strong decrease in the expression levels of Hsp90 after AngII treatment (Fig. 3C). This is accompanied by a significant increase in the expression of the eNOS disabling protein NOSTRIN (Fig. 5D), a protein that negatively modulates eNOS activity promoting the translocation of the enzyme from the plasma membrane to intracellular vesicles ([25] and references therein). Moreover, in both untreated (Fig. 5A) and AngII-treated (Fig. 5B) hearts, immunofluorescence localized NOSTRIN on the vascular endothelium of the greatest vessels (Fig. 5C) and on the ventricular EE, although at the endocardial level the signal appeared stronger in AngII treated hearts (Fig. 5B).

4. Discussion

In the present study we show, for the first time, that chronic (two months) treatment with AngII induces structural modifications of eel ventricular wall and myocardial vessels. This remodelling is paralleled by modification of NO production, and by changes in the expression and localization of molecules which regulate NOS activity, such as the protein kinase B (Akt), the eNOS disabling protein NOSTRIN, and the heat shock protein 90 (Hsp90).

With respect to the compact type of ventricular myoarchitecture typical of homeotherm hearts, the *mixed type ventricle* of the eel shows an outer layer of orderly and densely arranged bundles of muscle tissue (named *compacta)*, that enclose an inner spongiosa made up of a crisscrossed array of myocardial bundles (*trabeculae*) (see Ref. [26], for references). As revealed by morphological analyses, the cardiac ventricle of eels chronically exposed to AngII undergoes structural modifications, becoming more “muscularized” than untreated animals. This remodelling occurs at the level of both *compacta* and *spongiosa*. In particular, when compared to control animals, AngII-treated eels showed an increased *compacta* thickness, and a larger diameter of the trabeculae that form the *spongiosa*. These changes are accompanied by higher collagen

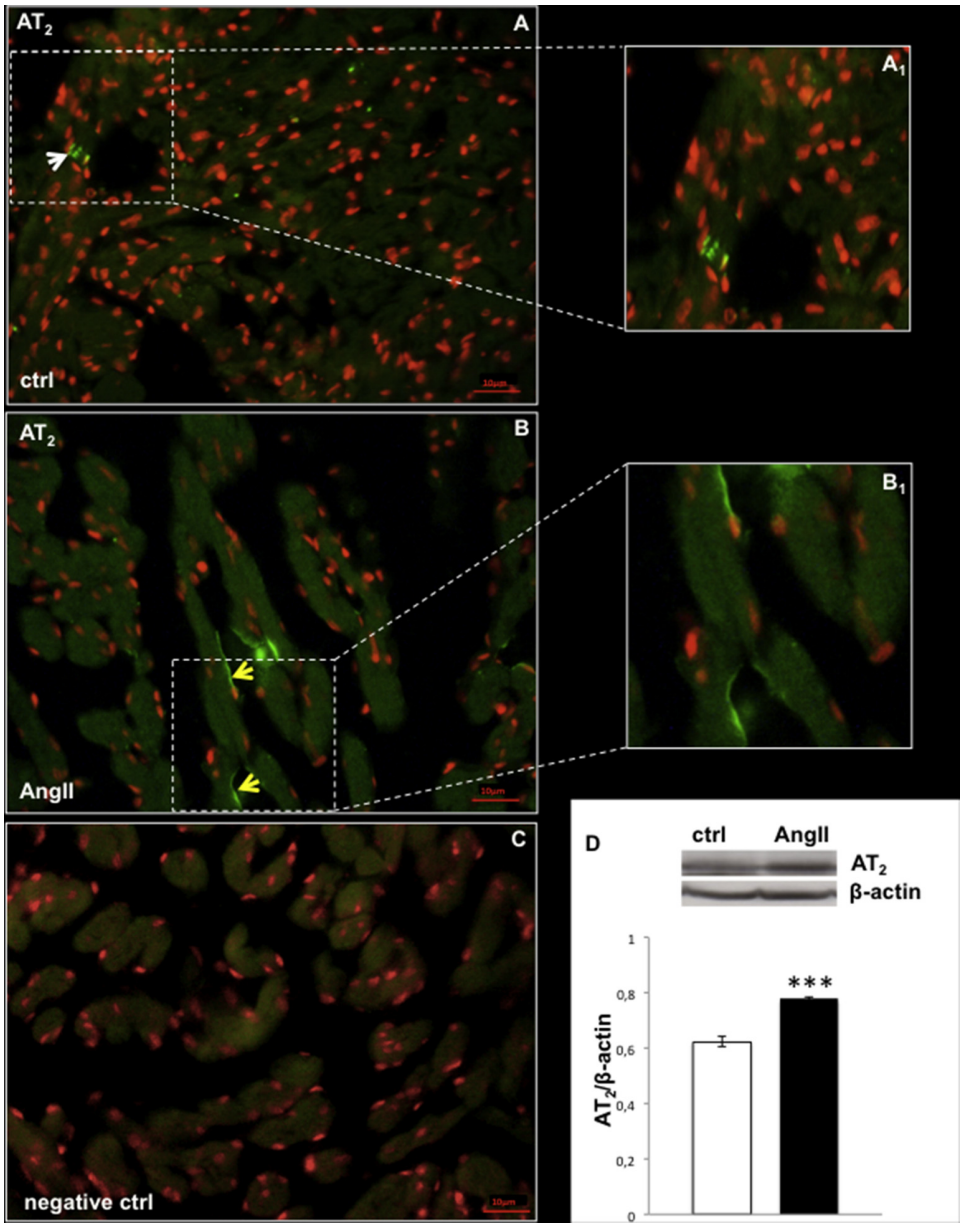

Fig. 2. (A, B) Representative images of AT_2 immunolocalization in the ventricle of control (A) and AngII treated (B) eels. White arrow: myocardiocytes. Yellow arrows: endocardial endothelium. Nuclei counterstaining: propidium iodide. Inset A_1 and B_1 represent a higher magnification of AT_2 positive signal. (C) Negative control. (D) Western blotting and densitometric analysis of AT_2 in cardiac extracts of ctrl and AngII treated eels. In D, loaded protein amount was verified using anti-β-actin antibody. Statistical differences were evaluated by unpaired *t-test* (***$p < 0.0005$). Data are the means ± SEM of 3 determinations for each group. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

deposition in the compact layer, shown by an intense Sirius red staining, particularly localized in the wall of subepicardial and coronary vessels. Enhancement of the compact myocardium is a strategy of cardiac growth that occurs in many fish species (*Ciprinus carpio*: [27]; *Salmo salar*: [28]; *Thunnus thynnus*: [29]; *Salmo gairdneri*: [30]). It was described in *A. anguilla* during ontogenetic growth [26] and in response to one month exposure to AngII [15]. Of note, we observed in eels treated for two months with AngII that the increment of the compact layer is accompanied by an increased vascularisation. In non-mammalian vertebrates, the growing heart increases capillarization of the compact myocardium through tightly controlled local mechanisms, which include mechanical (e.g. myocardial stretch), metabolic and growth factors such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) ([31] and references therein). Although the specific mechanisms that control eel heart vascularisation were not analysed in our study, it is conceivable that the enlarged vascular supply, which occurs in the *compacta* of the AngII-treated hearts, may allow the heart to cope with the metabolic and energetic demands of the deeper myocardial cells.

In mammals, AngII-dependent effects on myocyte growth correlate with the activation of the AT_1 receptor (see Ref. [32] for references). Contrarily, the AT_2 receptor, whose cardiac expression is reduced after birth, is generally reported to either offset or oppose the AT_1-induced effects on cell growth, thus mediating anti-growth and apoptotic actions [33]. However, Ichihara and co-

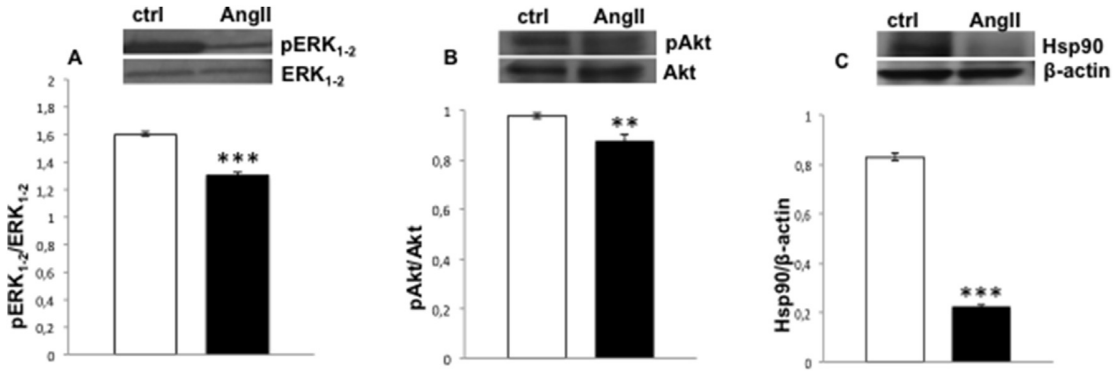

Fig. 3. Western blotting and densitometric analysis of $pERK_{1-2}/ERK_{1-2}$ (A), pAkt/Akt (B) and Hsp90 (C) in cardiac extracts of control and AngII-treated eels. In C, loaded protein amount was verified using anti-β-actin antibody. Statistical differences were evaluated by unpaired *t-test* (**p < 0.005; ***p < 0.0005). Data are the means ± SEM of 3 determinations for each group.

workers [34] reported in mice a role for AT_2 receptors in AngII-dependent cardiomyocyte hypertrophy and cardiac fibrosis. In *A. anguilla*, the few available data correlate the growth-promoting actions of AngII to the activation of a receptor able to bind mammalian anti-AT_2 antibody [15]. AT_1 receptors were not detected in control and AngII-treated eels. Conversely, western blotting and immunofluorescence showed, in the heart of the two groups of animals, a different expression and spatial localization of AT_2 receptors. In fact, while in control hearts AT_2 receptors are expressed in few myocardiocytes, after AngII treatment, they localize more widely in the EE. This suggests that, contrarily to mammals, in the eel heart AT_2 receptors participate to the mechanisms of remodelling induced by AngII. No data about a putative AngII-dependent modulation of ATs expression are available in fish. Also in mammals the molecular mechanisms responsible for the expression of the AT_2-R gene are not fully defined, although an upregulation of the AT_2 expression has been observed in different experimental models, also in response to AngII exposition, and in relation to various physio-pathological conditions (references in Ref. [35]). It is interesting to underline that, although eel AT_2 shares its origin with mammalian AT_2 [14], it underwent a peculiar evolution after divergence of its lineage [14]. This may explain the contrasting features showed by this receptor in mammals *vs* teleost, as for example the activation of second messengers [14], and thus the different elicited responses.

Several studies in mammals reported that AngII exerts its actions on the heart by recruiting ERK_{1-2} (see for references [36]). This kinase is known for its stimulatory role in the mechanisms of myocardial proliferation and fibrosis [37,38]. However, more recent experiments performed on *in vivo* knock-out mice showed that ERK_{1-2} signaling is not required for mediating cardiac hypertrophy [39].

In mammalian cardiomyocytes, AngII, *via* AT_1 and/or AT_2-receptor stimulation, exerts distinct effects on ERK_{1-2} activity. Through AT_1 receptor it may induce ERK_{1-2} activation, while it may inhibit ERK_{1-2}-dependent pathway through AT_2 receptor [40]. Consistent with these observations, we observed a significant reduction of the activated (phosphorylated) form of ERK_{1-2} in AngII-treated animals. Since this is accompanied by an increased expression of AT_2 receptors, it is possible that ERK_{1-2} down-regulation is mediated by the AngII-AT_2 binding.

An important aspect of the molecular network guiding the AngII-dependent cardiac morpho-functional modulation is the cross-talk with the NO signaling. In mammals, in addition to the AT_2-mediated control of NO production (references in Ref. [41]), AngII activates a number of signal-transduction pathways that result in reduced NO levels [42]. In turn, NO can counteract AngII-dependent effects by down-regulating the synthesis of both ACE and AT_1 receptors [5,6]. Thus, NO generation is both downstream and upstream the AngII-dependent cascade. At present, tissue levels of nitrite, an oxidative NO metabolite, represents a marker of constitutive NOS activity and consequently of NO generation [23]. By measuring total nitrite concentration in endothelial cell cultures, Li and co-workers observed an AngII-dependent reduction of eNOS-dependent NO production [43]. In the present work, we found a significant decrease in the ventricle nitrite concentration from 1.33 μmol L^{-1} in control animals (which compares with other fish species [44,45]) to 0.6 μmol L^{-1} in AngII treated eels. This suggests that chronic exposure to AngII is accompanied by a reduced NO generation, possibly mediated by a blunted NOS functionality. To analyse this possibility, we focused on the molecular modulation of NOS-dependent NO production, in particular the protein-protein interactions that represent important post-translational mechanism of eNOS. A variety of regulatory proteins cooperate to finely control eNOS activity. This is the case of calmodulin, Hsp90 and protein kinase B (Akt), that positively modulate eNOS [46]. At the same time, negative eNOS modulators include caveolin, and NOSTRIN [47,48]. The latter represents a crucial eNOS trafficking controller that, by binding the enzyme, triggers its translocation from the plasmalemma to vesicle-like subcellular structures, thus blunting eNOS-dependent NO production [48,49].

In teleosts, as well as in agnathans and chondrichthyans, a canonical eNOS seems to be absent (see Ref. [50]). Nevertheless, physio-pharmacological approaches, as well as NADPH-diaphorase and immunolocalization with heterologous mammalian antibodies, revealed the presence of an "eNOS-like" activity in the heart of several teleost species [18,24,51–55]. Ongoing studies will clarify this aspect. However, in the present study, by western blotting and immunofluorescence, we analysed whether and to which extent, the reduced NO generation observed in the eel heart after chronic exposure to AngII is associated with a modulation of eNOS controlling proteins, Akt, Hsp90 and NOSTRIN. Compared to the control, AngII-treated hearts showed comparable expression of peNOS-like enzyme, a slight decrease of p-Akt and a high decrement of Hsp90. Akt-mediated activation of eNOS is strongly influenced by the association between Hsp90 and the enzyme [56]. In fact, Hsp90 can either directly act as a scaffold factor between eNOS and Akt, or indirectly prevent Akt dephosphorylation from protein phosphatase 2A [57]. In addition, decreased levels of Hsp90 are responsible of a reduced displacement of caveolin-1 from eNOS [58], blunting enzyme activity. The bio-molecular results obtained on the

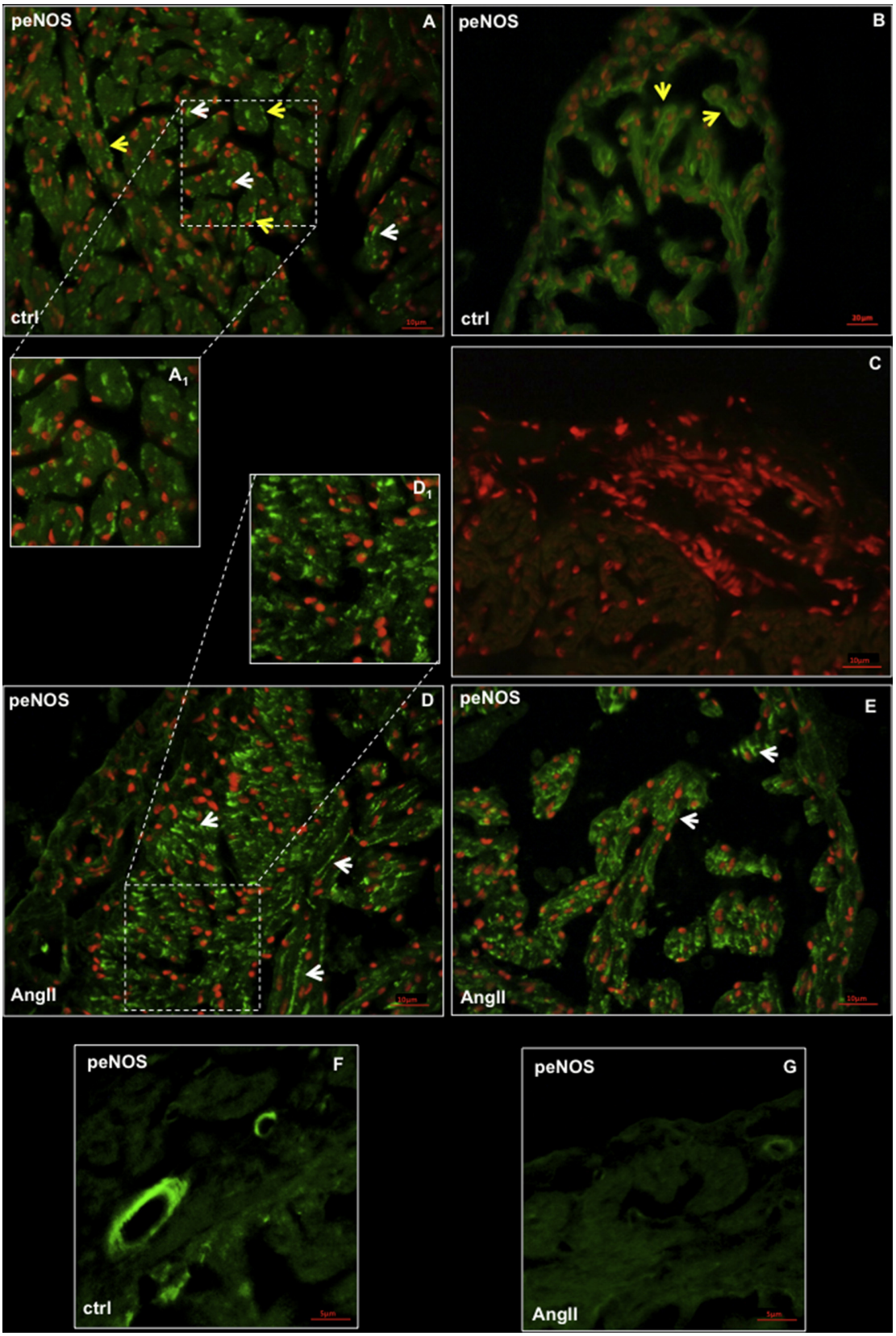

Fig. 4. Representative images showing peNOS immunolocalization on cardiac sections of control (A, B, F) and AngII (D, E, G) treated eels. (A, D) ventricle, (B, E) atrium, (F,G) vessels. Inset A_1 and D_1 represent a higher magnification of peNOS positive signal. (C) Negative control. White arrows: myocardiocytes. Yellow arrows: endocardial endothelium. Nuclei counterstaining: propidium iodide. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

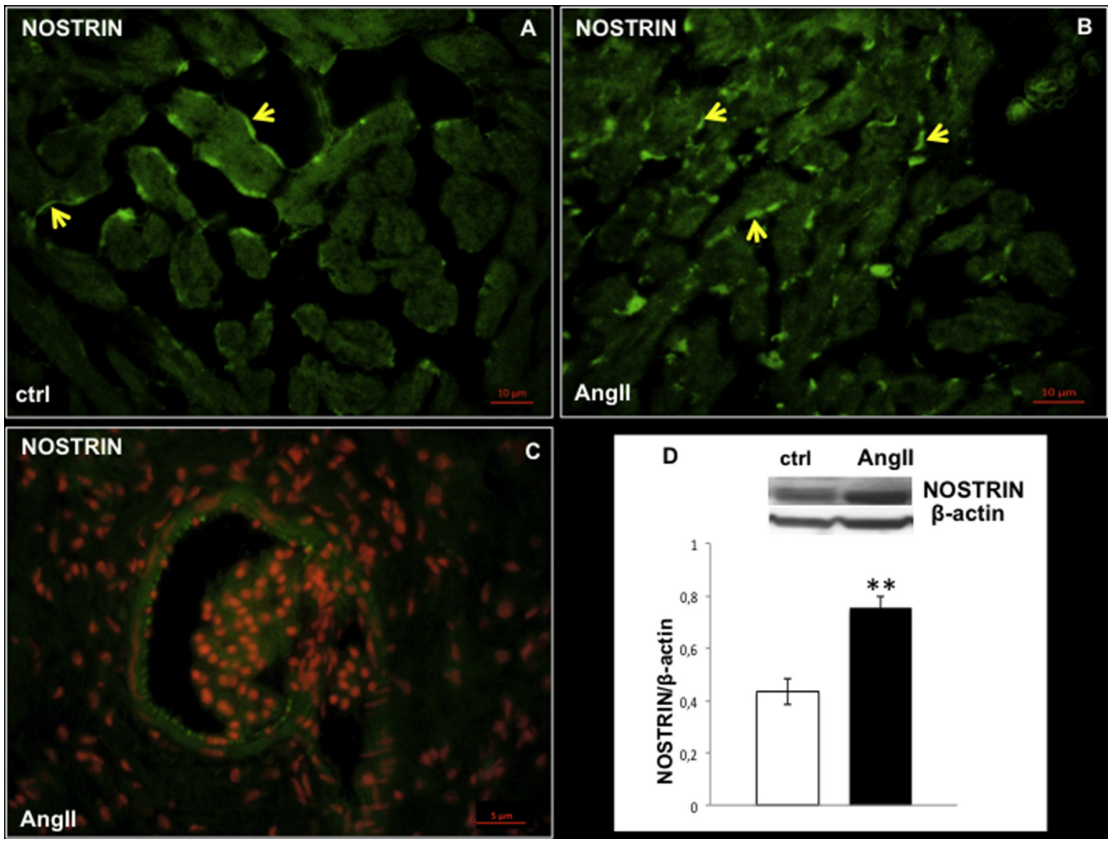

Fig. 5. Representative images of NOSTRIN immunolocalization in ventricular sections of control (A) and AngII-treated (B, C) eels. NOSTRIN localized in endocardial (yellow arrows) and vascular endothelium (red arrows). C: nuclear counterstaining with propidium iodide. (D) Western blotting of NOSTRIN in cardiac extracts of control and AngII treated eels. Loaded protein amount was verified using anti-β-actin antibody. Statistical differences were evaluated by unpaired *t-test* (**$p < 0.005$). Data are the means ± SEM of 3 determinations for each group. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

remodelled heart of AngII-treated eels suggest that long-time exposure to the hormone may induce a decrease of eNOS-like activity by modulating the expression of its intracellular regulators Akt and Hsp90. Of note, in AngII-treated hearts, we also found an enhancement of ventricular NOSTRIN expression. To the best of our knowledge, this is the first evidence revealing the presence of this protein in the teleost myocardium. In mammals, NOSTRIN binds eNOS to induce its translocation and inactivation [48]. This binding is selective for this NOS isoform. In the light of the debate about NOSs characterization in teleost (see above), the presence of an eNOS-selective binding partner in the eel heart supports the possibility that the piscine isoform is an eNOS-like enzyme. In parallel with NOSTRIN detection, by immunofluorescence we found on ventricular sections of both untreated and treated eels that the protein localizes on the vascular endothelium of greater vessels and on the EE. In particular, in treated eels, the EE was characterized by a higher NOSTRIN signal and a reduced peNOS localization. If, as in mammals (see Ref. [59] for references), also in the eel heart a higher NOSTRIN expression is responsible for a reduction of NO production through enzyme inactivation requires further investigations.

Although in the present study we mainly focused on myocardial and endocardial tissues, immunofluorescence revealed that, also in large vessels, chronic AngII treatment is accompanied by a reduction of peNOS signal that resemble those occurring on endocardial cells. The functional significance of these results in relation to the AngII-mediated influence on cardiac remodeling in teleost was unexplored in the present study. However, extensive evidence exists in mammals on the relationship between AngII and the NOS/NO system on both normal and pathological vascular homeostasis. It was shown in mammals that AngII induces an increase in endothelial oxidative stress, with subsequent adverse effects on vascular function and a downregulation of eNOS activity (see Ref. [60] for references). Thus, eNOS modifications induced by AngII affect the downstream NO activated signaling pathways with effects on the vascular tone. As shown in mammalian aortic rings, AngII decreases endothelium-dependent relaxation, and this in part contributes to endothelial dysfunction (references in [61]). Based on these observations it is possible to speculate that in the eel heart, chronic Ang-II treatment, by reducing vascular NO generation, may negatively affect vascular relaxation, and thus the perfusion of the compact myocardium where larger vessels are mainly located. Whether and to which extent the AngII-dependent ventricular rearrangement as well as the related reduction of NO production at both myocardial and vascular level may be beneficial or detrimental for the eel heart at the moment is unknown and remains a topic for future research.

5. Conclusion

In conclusion, the present study provides large evidence that the eel heart undergoes structural and molecular remodelling if exposed for two months to the cardioactive hormone AngII. This occurs through molecular mechanisms which involves modification of NO production, and changes in the expression and localization of molecules which regulate NOS activity. Although the specific NOS isoform remains to be defined, our results strongly

suggest that in the eel the cardiac NOS/NO system acts as an important coordinator/integrator of molecular signal cascades which control cardiac form and function.

Future research will contribute to better decipher the complex molecular neuro-humoral networks which control cardiac remodelling in fish. In this context, the use of animal models, such as the eel, characterized by a remarkable morpho-functional adaptability to a variety of environmental factors (temperature, partial pressure of oxygen, pH, environmental pollutants, etc.) [19] could be of help. In an evolutionary and comparative perspective, we hope that the information on the molecular strategies of cardiac regulation in fish will cross the boundaries of comparative, integrative and environmental physiology, emerging also as a topic of interest and a source of bioinspiration for human cardiovascular physiology.

Conflict of interest

None.

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REVIEW

ACTA PHYSIOLOGICA

NO, CO and H_2S: What about gasotransmitters in fish and amphibian heart?

S. Imbrogno | M. Filice | M. C. Cerra | A. Gattuso

Department of Biology, Ecology and Earth Sciences, University of Calabria, Arcavacata di Rende, Italy

Correspondence
M. C. Cerra and A. Gattuso, Laboratory of Cardiovascular Physiology, Di.B.E.S.T. – University of Calabria, Arcavacata di Rende, Italy.
Emails: maria_carmela.cerra@unical.it, alfonsina.gattuso@unical.it

Abstract

The gasotransmitters nitric oxide (NO), carbon monoxide (CO), and hydrogen sulphide (H_2S), long considered only toxicant, are produced in vivo during the catabolism of common biological molecules and are crucial for a large variety of physiological processes. Mounting evidence is emerging that in poikilotherm vertebrates, as in mammals, they modulate the basal performance of the heart and the response to stress challenges. In this review, we will focus on teleost fish and amphibians to highlight the evolutionary importance in vertebrates of the cardiac control elicited by NO, CO and H_2S, and the conservation of the intracellular cascades they activate. Although many gaps are still present due to discontinuous information, we will use examples obtained by studies from our and other laboratories to illustrate the complexity of the mechanisms that, by involving gasotransmitters, allow beat-to-beat, short-, medium- and long-term cardiac homoeostasis. By presenting the latest data, we will also provide a framework in which the peculiar morpho-functional arrangement of the teleost and amphibian heart can be considered as a reference tool to decipher cardiac regulatory networks which are difficult to explore using more conventional vertebrates, such as mammals.

KEYWORDS

carbon monoxide, cardiac modulation, hydrogen sulphide, nitric oxide, poikilotherms, signal transduction

1 | INTRODUCTION

The term "gasotransmitter" was firstly proposed by Wang[1] to indicate a signalling molecule that is enzymatically synthesized endogenously, is membrane permeable without binding to a receptor and has specific cellular and molecular targets, and its effects may or may not be mediated by second messengers.[1]

The exploration of the biological properties of gaseous molecules, generated in vivo by living organisms during catabolic processing, was prompted by the identification of nitric oxide (NO) as the endothelium-derived relaxing factor.[2,3] This finding, which revealed for the first time that a gas may carry regulatory signals to a given cell, opened a novel era of intense investigations which resulted in the identification of the functional importance of other 2 gasotransmitters, carbon monoxide (CO) and hydrogen sulphide (H_2S), and now is going to propose ammonia and methane as novel candidates (for references, see ref. 4). As summarized in the "SAVE" acronym (Simplicity, Availability, Volatility, Effectiveness), NO, CO and H_2S share major features.[4] They are small and very simple molecules whose generation does not require complicated metabolic pathways and multiple substrates. They can easily cross cell membrane and use the blood to move in the organism only by covalent interaction with several "carrier" proteins. Their volatile nature induces a rapid turn-off of the signalling they activate. Lastly, their ubiquitous presence is the prerequisite for a large variety of functions that result in a fine-tuned global preservation of whole organism homoeostasis.

NO is produced by the family of intracellular enzymes named nitric oxide synthases (NOSs) that, in the presence of oxygen, use L-arginine as substrate.[5] A huge effort of research from many laboratories has now extensively documented its crucial physiological relevance in almost all living organisms. Also CO is enzymatically generated within the cell during the haem metabolism by haem oxygenase-1 and 2 (HO-1 and HO-2). It shares several functions with NO, as in the case of vascular regulation and protection against ischaemia/reperfusion (I/R) damages.[6] H_2S, the last gasotransmitter to be identified, is generated in various mammalian cells *via* the enzymes cystathionine β-lyase (CBE), cystathionine γ-lyase (CSE) and 3-mercaptopyruvate sulfurtransferase (MST)/cysteine aminotransferase (CAT) that use L-cysteine and homocysteine or their derivatives as substrates.[7] H_2S mainly acts as a neurotransmitter and a neuromodulator, but it contributes to regulate many other functions, including cardiovascular performance.[8]

So far, because of putative biomedical implications, a huge literature on gasotransmitters has been mainly accumulated in mammals, and excellent reviews have been published on this topic. However, recent effort of comparative research revealed that NO, CO and H_2S evolved as early regulatory molecules in non-mammalian vertebrates before the establishment of complex animal body plans, as those encountered in mammals. In poikilotherms, these gasotransmitters are emerging as crucial modulators of many physiological processes, including basal organ performance, adaptive response to stress, vascular regulation, neural plasticity, respiratory control and organ development.[9-12] In addition, many poikilotherms are adapted to habitats in which NO, CO and H_2S represent an environmental hazard, being present at high concentrations. For these organisms, gasotransmitters represent relevant physiochemical stressors that animals tolerate thanks to an impressive functional plasticity.

With respect to conventional laboratory species (eg rodents), animals such as fish, amphibians and reptiles have always been used by physiological research to describe complex systems and processes whose importance is well established in the phylogenetically more advanced mammals (for extensive review and references, see ref. 13). This is epitomized by the pioneering studies by Otto Loewi that, using the frog heart, in the early 1900s initiated the story of the adrenergic and cholinergic cardiac control in vertebrates, whose outcomes include the seminal identification of NO as the endothelial mediator of the acetylcholine (ACh)-dependent vasomotility control (for references, see ref. 14).

In poikilotherm vertebrates, the heart is a clear example of organ morpho-functional flexibility. As largely documented in fish and amphibians,[15-21] it supports the varying requirements of the animal by adjusting metabolism and haemodynamics in response to extrinsic and intrinsic stimuli, including circulating and intracardiac hormones and humoral autacoids (ie locally generated signalling substances).[22] Under normal and stressful conditions, these substances modulate the heart acting beat-to-beat (Frank-Starling response), as well as in the short (myocardial contractility, excitation-contraction coupling)- and long-term (gene expression).[14] In this context, locally released autacoids, such as NO, CO and H_2S, play a remarkable role. This is exemplified by the NOS/NO system that, in teleost fish and amphibian heart, acts as a major integrative mechanism that coordinates humoral signals and the crosstalk between the different cardiac regions (ie the endocardial endothelium [EE] and the myocardium), thus contributing to the morphological and functional integration which is essential for proper organ function.[22,23]

Based on these premises, and using the predominant mammalian literature as a reference point, we designed this review to analyse, in a comparative perspective, the modulation elicited by NO, CO and H_2S on the cardiac function of fish and amphibians. For the reader who is not familiar with the functional morphology of the heart of fish and amphibians, and with the pertinent anatomical terminology, we will also briefly summarize some basic traits related to the issue of this review.

2 | CARDIAC DESIGN IN FISH AND AMPHIBIANS

In fish and amphibians, the heart shares structural and functional aspects with that of other non-mammalian vertebrates and with that of mammals, but it remarkably differs in terms of myoarchitecture, vascular supply and pumping performance.

The fish heart, shaped by 4 chambers in series (*sinus venosus*, atrium, ventricle, and outflow tract or *bulbus cordis*), represents the prototype of the higher vertebrate heart. It pumps the venous blood to the gills to be oxygenated and then distributed to the body, then reflowing to the heart.

Among vertebrates, teleost fish show the highest intraclass variation in heart ventricle myoarchitecture. According to the classification proposed by Tota,[24] the cardiac organization in fish spans from a fully trabeculated ventricle to a mixed type (Figure 1). The trabeculated ventricle is made up of a crisscrossed array of myocardial bundles (*trabeculae*) that give the ventricular wall a spongy appearance (ie *spongiosa*), and is supplied by the superfusing venous blood that circulates through the intertrabecular spaces (la*cunae*). In the mixed type, the numerous *trabeculae* of the avascular *spongiosa* are lined by a thin layer of EE which separates the ventricular lumen into an extensive network of *lacunae* of different size. The *spongiosa* is

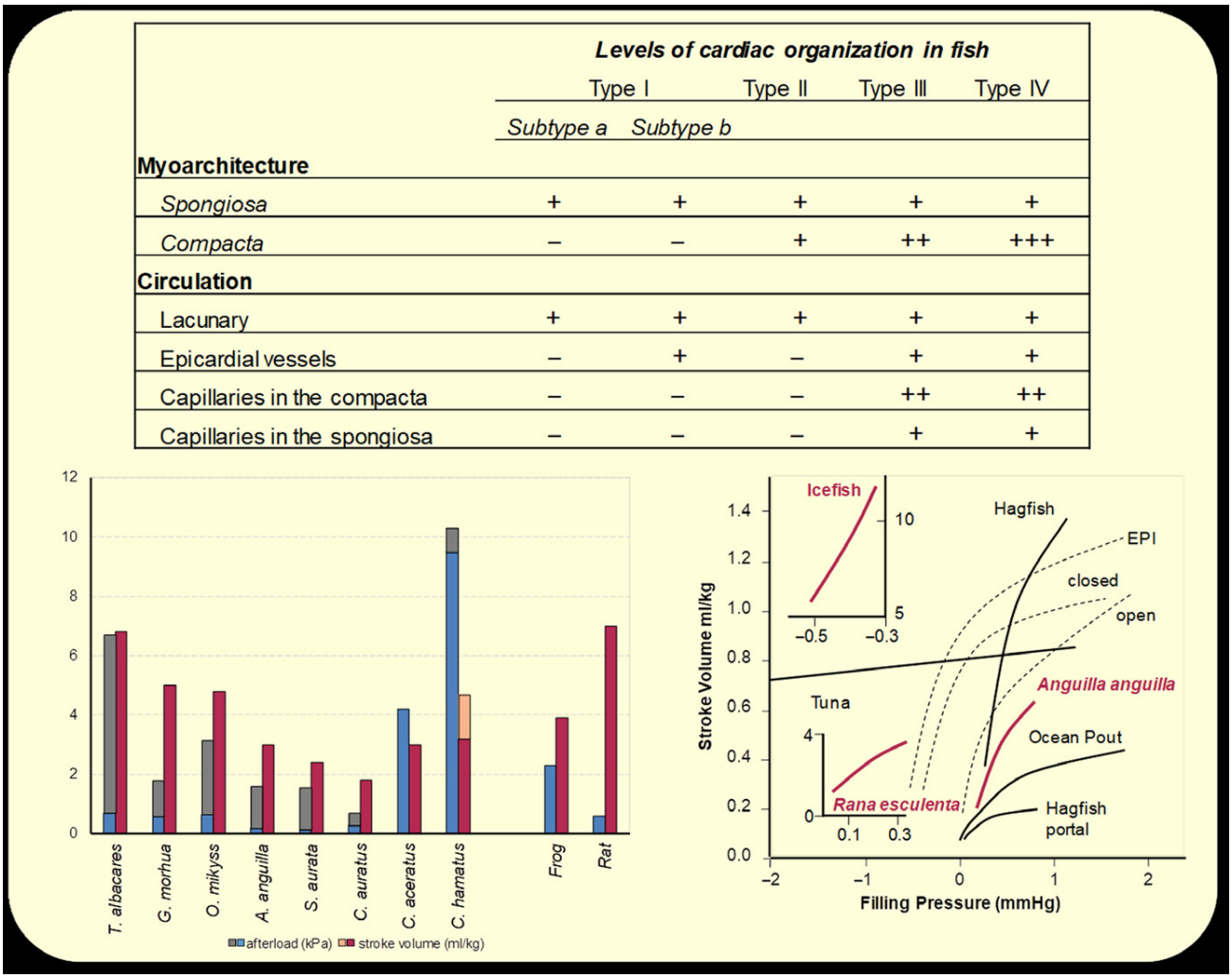

FIGURE 1 *Top panel*: different levels of cardiac organization in fish (modified from ref. 24). *Low panel (left)*: values of afterload (blue columns) and stroke volume (red columns) in fish, frog and rat under in vitro basal or in vivo resting conditions. The increases in afterload (grey columns) and stroke volume (orange column) under conditions of maximal stroke work (ie measured in vivo during exercise or in vitro under maximum sustainable output pressure) are also illustrated (modified from ref. 26). *Low panel (right)*: Starling curves obtained in in vitro hearts of *R. esculenta* and several fish. Dashed lines are related to curves obtained in *O. mykiss* in opening/closed pericardium preparations and in the presence of adrenaline (ADR) (modified from ref. 190)

covered by an outer layer of orderly and densely arranged myocardial bundles, named *compacta*, perfused by an arterial supply.[24,25] In fish, the different types of ventricular myoarchitecture are associated with a different mechanical behaviour expressed in terms of pressure generation and stroke volume, so that the fish heart can work as either a volume or a pressure pump. The extreme haemodynamic conditions are represented by icefish and tuna. The first belongs to the highly trabeculated type I heart ventricle. Being able to pump large end-diastolic volumes at relatively low pressures (high ventricular compliance), it represents a prototype of volume pump. Of note, the volume pump type of ventricle is characterized by a high sensitivity to increased filling pressure changes. Thus, the Frank-Starling response, which in fish represents a major mechanism to regulate cardiac output, allows this heart to displace very high stroke volumes.[26] On the opposite, the tuna heart, equipped with very thick *compacta*, belongs to type IV. Its haemodynamic behaviour is represented by relatively small stroke volumes at high rate and high pressures, thus representing a typical pressure pump[26] (Figure 1).

In amphibians, the heart possesses a *sinus venosus*, 2 atria divided by an anatomically complete internal septum, a ventricle without internal subdivision and a *conus arteriosus* with a spiral valve. In the single ventricle, the "venous" and the more "arterial" blood remains to a large extent unmixed, being distributed, during different systolic

phases, in the low-pressure pulmocutaneous arch (venous blood), in the systemic arches (mixed blood) and in the high-resistance carotid arches (for references, see ref. 14). In the frog heart, considered as a paradigm of the amphibian heart, the single ventricle consists of a *spongiosa* supplied by myriads of *lacunae* extending through the ventricle wall to the epicardial surface. As the wall contains no capillaries, the entirely trabeculated ventricle shows an impressively large surface area of the EE that covers the luminal cavity.[27]

Of remarkable functional importance, in both fish and amphibians, the extensive EE surface area is crucial for mediating the communication between blood-born stimuli and the subjacent myocardium. It also plays an important role in the intracavitary regulation of the cardiac performance.[14] Thanks to the presence of a variety of receptors for endocrines and growth factors, the EE of fish and amphibians acts as a paracrine-autocrine-endocrine transducer and a sensor-integrator device. As illustrated in this review, this is exemplified by the NOS/NO-mediated signalling located on the EE that, in the absence of vascular supply, is mandatory for modulating both basal cardiac performance and the adaptive response.

3 | NOS/NO SYSTEM

NO was the first gas to be identified as a biologically relevant molecule. In almost every animal tissue, it is generated by the family of NOS isoenzymes, that is the constitutive endothelial (eNOS/NOS3) and neuronal (nNOS/NOS1) isoforms, and the inducible isoform (iNOS/NOS2). They use L-arginine as a substrate in combination with O_2 to generate NO. In the steady state, nanomolar concentrations of the gas are produced. However, when iNOS is activated by immunological and inflammatory stimuli, micromolar cytotoxic amounts are generated.[28] As largely shown in mammalian cells, the spatial compartmentalization of NOS isoenzymes allows NO to be generated in proximity of its molecular targets and this compensates for its very short half-life (2 milliseconds to 2 seconds in vivo;[29] 1-30 seconds in vitro[30]). NO is rapidly metabolized in the relatively more stable metabolites, such as nitrite, nitrate, iron-nitrosyl, S-nitroso and N-nitroso compounds.[31] Under hypoxic and/or acidic conditions, nitrite can be converted to NO by a number of cellular proteins, including deoxygenated haemoglobin (Hb), myoglobin (Mb), neuroglobin, xanthine oxidoreductase and carbonic anhydrase (for references, see ref. 31).

Two major mechanisms mediate the NO effects. One is soluble guanylate cyclase (sGC)–dependent; the other is sGC-independent. The interaction with sGC stimulates the generation of the second messenger cGMP that, in turn, activates cGMP-dependent protein kinase (PKG), cGMP-gated cation channels and cGMP-modulated phosphodiesterases (PDE2). This initiates a number of processes that involve many intracellular targets.[32] Alternatively, NO can by-pass sGC by directly interacting with other haem proteins and non-haem iron.[33] In addition, NO can react with cysteine thiol residues forming a S-nitrosothiol.[34] This reversible NO-dependent post-translational protein modification, called S-nitrosylation, involves many intracellular targets allowing for a wide range of NO-mediated functions.[35]

The heart is a major NO producer, being at the same time, a target of its actions. As here following illustrated, NO, generated by myocardial and non-myocardial tissues, elicits an autocrine-paracrine control of beat-to-beat, short-term and long-term cardiac responses. Importantly, thanks to its neurotransmitter properties and the regulation of major intracellular second messengers of cholinergic and adrenergic signals (ie cGMP and cAMP), the NOS/NO system is crucially located at the interface between the heart and its autonomic control. Lastly, as later described in this review, it represents a mediator of many endocrine signals converging to the heart.

4 | NOS CARDIAC EXPRESSION AND LOCALIZATION

4.1 | Mammals

The mammalian heart expresses both constitutive and inducible NOSs. eNOS is present in coronary and EE cells and cardiomyocytes. In these cells, it is typically expressed into the caveolar microdomains together with important components of NO-modulated signal transduction pathways (eg β-adrenergic receptors [ARs], cholinergic receptors and L-type Ca^{2+} channels; reviewed in ref. 36). nNOS is expressed in cardiac autonomic nerves and ganglia. It is also present in cardiomyocytes at the level of the sarcoplasmic reticulum (SR)[37] and bound to sarcolemmal membrane proteins.[38] Under pathological conditions, as in the case of septic shock[39] and heart failure,[40] iNOS is induced in both cardiomyocytes and inflammatory cells infiltrating the myocardium. In addition, beyond this established pathophysiological trait, the identification of a constitutive iNOS suggests that the enzyme plays a role also under normal conditions.[41] A mitochondrial NOS (mtNOS), corresponding to a variant of nNOS, has been reported in cardiomyocytes, expanding the intracardiac pool of NO-generating mechanisms.[42]

4.2 | Fish

A number of functional and biochemical studies has established that the fish heart expresses a functional NOS/NO

system, deeply involved in the organization of basal haemodynamics,[43-45] as well as of the response to physical[16,43-45] and chemical challenges.[21,43,46-51] However, despite a remarkable research effort, several aspects of the piscine NOS/NO system remain unresolved. A major conflicting point is the characterization of the tissue NOS equipment, particularly in relation to the questioned presence of a canonical eNOS. In fact, the discontinuous identification of a piscine eNOS sequence, the use of heterologous mammalian antibodies for immunohistochemical studies to demonstrate apparent eNOS expression[52-54] and the major role of neuronal-derived NO in classic NO-dependent vasomotor response suggest the enzyme is not expressed, at least in teleost.[55] Based on comparative genomic analyses, and information from fish genome database, it is proposed that only 2 NOS isoforms exist in fish, that is nNOS and iNOS. In some species (eg the cyprinids zebrafish and goldfish), 2 iNOS isoforms are expressed, probably as a consequence of the third round of genome duplication that occurred in teleost fish. Interestingly, although phylogenetic analysis regroups the constitutive nNOS and eNOS separately from vertebrate iNOS,[56] many iNOS sequences of cyprinids possess a myristoylation site, which is characteristic of eNOS.[57] At the same time, in fish, a nNOS isoform, that includes an endothelial-like consensus, may cover some functional behaviours typical of the eNOS isoform identity.[57] The discovery of a putative nos3 gene in actinopterygian recently suggested that the nos1 gene must have duplicated early in gnathostome evolution, before the actinopterygian-sarcopterygian split, but after the appearance of the chondrichthyan fish lineage.[58] Despite the above data, studies mainly performed by our research group, using physio-pharmacological approaches, as well as NADPH-diaphorase and immunostaining with heterologous mammalian anti-eNOS antibodies, show the presence of an "eNOS-like" activity in the heart of several teleost species.[17,21,50,59] These species belong to distinct phylogenies and ecophysiological habitats. They include extreme stenotherms such as the teleosts endemic of Antarctic waters (ie the red-blooded *Trematomus bernacchii,* the Hb-free *Chionodraco hamatus* and the Hb-free/Mb-free *Chaenocephalus aceratus*), and the temperate eurytherms *Thunnus thynnus thynnus* and *Anguilla anguilla*. Immunological evidence for eNOS expression is also reported in 2 ancient fish, the aestivating sarcopterygian dipnoi (lungfish) *Protopterus dolloi* and *Protopterus annectens.*[60,61] This is of relevance as, even if nos3 sequence has not been identified in dipnoi, lungfishes possess many tetrapod-like gene sets (for references, see ref. 58) which may account for the nos3 presence in these fish. In all piscine species, the enzyme localizes at the EE which lines the ventricular trabeculae and, to a lesser extent, in both atrial and ventricular myocardiocytes, and at the level of the visceral pericardium. Regardless of the isoform type, this localization is indicative of a tissue-selective intracardiac production of NO. In fish cardiac designs characterized by a scarcely present, or even absent, coronary system (eg the teleost *A. anguilla*), this NO generation contributes to regulate the cardiac function acting as a local modulator and/or a neuro-visceral integrator. If the coronary circulation is present and well developed (eg the rainbow trout *Oncorhynchus mykiss*), NO also affects vascular resistance both in vivo[62] and in vitro.[63,64]

4.3 | Amphibians

Like in fish, there is still controversy concerning the presence of different NOS isoforms in amphibians, and, also in this case, the question on a canonical eNOS is still unresolved. A vascular endothelial NO system was demonstrated in the bullfrog *Rana catesbeiana*[65] and the leopard frog *Rana pipiens,*[66,67] but neither in the toad *Bufo marinus,*[68] nor in the clawed frog *Xenopus tropicalis*.[56] However, very recently, nos3 mRNA expression was found in the aortic endothelial cells of *Rhinella marina* (GenBank accession no. GU138862.1),[69] and, in contrast to previous studies,[56] also in the aorta of *X. tropicalis* and *Xenopus laevis*.[69] These data are of relevance in relation to the question about eNOS evolution. In fact, they suggest to reconsider the hypothesis that eNOS signalling firstly evolved in amniotes, as proposed by the identification of the enzyme also in reptiles.[58] Accordingly, amphibians can be considered as a transition phase in the evolution of eNOS expression in the cardiovascular system of tetrapods.[69]

In the heart, data obtained in *R. catesbeiana* suggest nNOS as the major cardiac isoform, particularly expressed in atrial intracardiac ganglion.[70] However, using anti-eNOS antibodies, immunoreactivity was observed on the EE of *Rana esculenta.*[71] Adler et al[72] proposed the EE as a major site for eNOS localization in the frog heart. In line with this, the presence of phosphorylated eNOS (pSer1177-eNOS), mainly located in the EE and, to a lesser extent, in myocardial and epicardial cells, was documented by immunostaining in *R. esculenta*.[73] More recently, a protein named "XteNOS," with a high sequence identity with mammalian eNOS was detected in the heart of *X. tropicalis*. This eNOS-like protein is present in the EE but not in the vascular endothelium, confirming its tissue-specific expression.[68]

5 | NO AS A CARDIAC MODULATOR: MAMMALS VS POIKILOTHERMS

NO control of the cardiac function has been particularly studied in relation to the excitation-contraction coupling. In

mammals, both exogenous (by NO donors) and endogenous NO modulates contractile state of cardiomyocytes in a concentration-dependent biphasic manner. At low concentrations, the gas exerts a positive action, while at higher concentrations it induces a negative effect (for references, see ref. 74). Also in poikilotherms, NO influences the cardiac contractility with effects that are species-specific and dependent on the experimental conditions.[22,23] It is now well recognized that the specific subcellular localization and regulation of the different NOS isoforms, and of NO target proteins allow NO to achieve a spatio-temporal control of different cellular functions.[75] As shown in mammals and in poikilotherms, each NOS isoform generates trace amounts of NO which acts in an autocrine-paracrine fashion to modulate the heart beat-to-beat, and in the short-, medium- and long-term.[23,76]

Generated within a cell, NO rapidly diffuses to modulate adjacent cells *via* paracrine effects. Interestingly, the very high EE surface-to-myocardial volume ratio of the poikilotherm heart offers an extraordinary opportunity for exploring the paracrine role of cardiac NO (for references, see refs 23,77). The extensive EE is an important source of the gas that acts as a freely diffusible messenger to modulate the performance of the subjacent myocardium (Figure 2). Consistent with this, in fish and amphibians, the functional alteration of the EE (eg by exposure to the detergent Triton X-100) increases myocardial contractility due to the impaired EE-NOS communication, which prevents the basal NO depressive tone (for references, see ref. 23). Interestingly, the EE of the poikilotherm heart is also crucially involved in coupling the NO system with endoluminal chemical stimuli (Figure 2). This role is supported by data in both teleost and amphibians. In the eel *A. anguilla,* in vitro perfusion techniques showed that an intact EE-NOS system is mandatory for the stimulation of contractility elicited by nanomolar concentrations of intraluminal ACh,[43] and for the negative effect induced by angiotensin II (AngII),[46] and the chromogranin-A (CgA)–derived vasostatin-1 (VS-1).[47,78,79] In the in vitro perfused *R. esculenta* heart, both positive and negative effects elicited by ACh on contractility are mediated by NO and involve the EE.[80] Also the cardiodepression induced by BRL_{37344}-dependent β_3-AR activation[81] and by low concentrations (0.01 nmol/L) of endothelin-1 (ET-1) requires the recruitment of the EE/NOS/NO pathway.[82] In the second case, this occurs *via* activation of endothelial ETB receptors.[82] Furthermore, the EE-NO signalling is obligatory in mediating the cardiodepression elicited in *R. esculenta* by the CgA-derived peptide catestatin,[20,79,82] as the EE impairment by Triton X-100 abolishes the NO-dependent effects of the peptide. Interestingly, in the isolated and Langendorff-perfused rat heart, catestatin-dependent

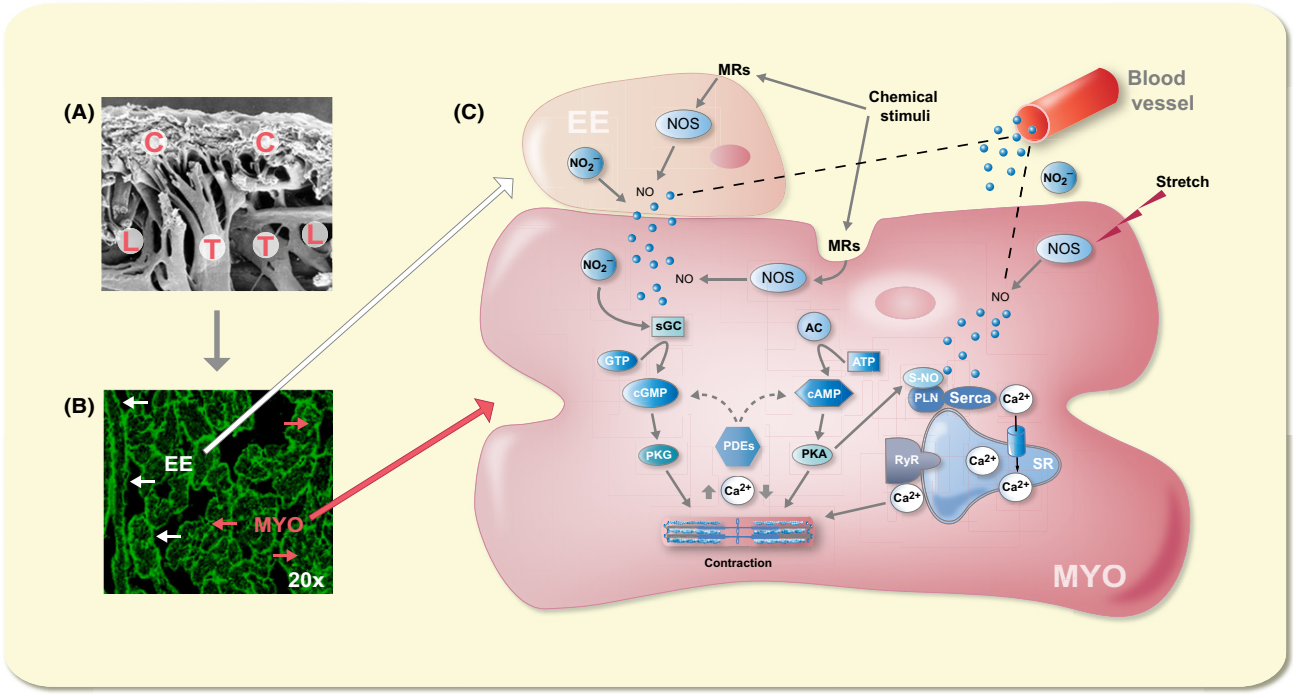

FIGURE 2 A, Scanning electron microscope image of the *A. anguilla* ventricle; L: lacunary spaces; T: *trabeculae*; C: *compacta*. From ref. 15. B, Representative image of ventricular eNOS immunolabelling in *A. anguilla*. White arrow: EE; red arrow: cardiomyocytes. C, Schematic diagram showing the role of NO as a spatial paracrine/autocrine/endocrine integrator. For details and abbreviations, see the text. Modified from ref. 23

depression of contractility and relaxation occurs *via* activation of the nitrergic cascade and requires an intact coronary endothelium.[83] The above observations underline the evolutionary importance of the NOS/NO system located at the endothelial interface between the blood and the myocardium. In the vascularized heart of mammals and several piscine species (ie tuna), this interface is represented by the vascular endothelium, while in the absence of coronary vasculature (avascular fish and frog ventricle), it is functionally represented by the EE. In both cases, the NOS/NO system appears a conserved trait.

Interestingly, a paracrine role of the NOS/NO system has been proposed in fish in relation to the cardiac adaptation to extreme environmental requirements. In the heart of the lungfish *P. dolloi*[60] and *P. annectens,*[61] eNOS immunodetection showed that during the first days of aestivation, the enzyme is upregulated. This suggested that as in the mammalian "short-term hibernating myocardium" (ie the metabolic adaptation to reduced oxygen), also in lungfish eNOS may contribute to myocardial protection against the ischaemic-like conditions induced by aestivation. This may occur through the involvement of the EE/NOS/NO system in the neuroendocrine regulation of the heart during the aestivation-dependent metabolic depression (for references, see ref. 60). In line with this, in the heart of the African lungfish *Protopterus aethiopicus,* NO generation was found to reduce myocardial oxygen consumption, and thus myocardial efficiency, and this may be of relevance for the aestivating animal.[84]

In parallel with the paracrine activity, NO contributes to cardiac modulation thanks to its autocrine properties, largely explored in mammals, but also documented in non-mammalian species. Autocrine NO is involved in the stretch-induced increase in contractility that occurs during the Frank-Starling response. This effect requires a spatio-temporal activation of eNOS and nNOS.[16,75,85] During the early phase of the myocardial response to enhanced preload, autocrine NO, generated by nNOS located on the SR, regulates intracellular Ca^{2+} by modulating the phospholamban (PLN)-SERCA2a complex. This occurs *via* a cGMP-independent activity. During the subsequent slow phase, when the myocardial fibres are stretched, activated eNOS sustains the late increase in the Ca^{2+} transient and force generation through a mechanism which involves S-nitrosylation of thiol residues of the ryanodine receptors Ca^{2+} release channels.[40,86] In this mechanism, also PKG has been recently proposed to play a role.[87]

As in mammals, in fish and amphibians, NOS spatial confinement is crucial in coordinating signalling networks between cardiac NO and specific intracellular effectors, allowing local control of different cellular functions. Research from our laboratory has documented in teleost species, such as the European eel *A. anguilla*[16] and the Atlantic salmon *Salmo salar,*[44] that autocrine NO contributes to the stretch-induced response. In the working eel heart, the NO-dependent increase in myocardial sensitivity to preload occurs bypassing the classic NO-dependent sGC/cGMP signalling cascade, but requires a modulation of the SERCA2a pump *via* S-nitrosylation of PLN (Figure 2).[16,88]

Also in amphibians, endogenous basal NO affects cardiac dynamics. This occurs by influencing the contraction/relaxation cycle, with important effects on ventricular pump mechanics.[73] When the isolated and perfused working frog (*R. esculenta)* heart is exposed to stretch challenges, NO release is increased and this modulates the relation between contraction and relaxation by acting on Ca^{2+} release from sarcolemma and on its removal by the SR.[19] Unlike the S-nitrosylation detected in the eel, these events require a cGMP-dependent mechanism.[19]

Despite the limited data in poikilotherms, the above observations suggest that the mechanisms of cardiac modulation, occurring through spatially restricted NO generation, are evolutionary conserved. This is supported by data on reptiles, as exemplified by observations in the turtle *Trachemys scripta*, in which autocrine NO, generated by exogenous L-arginine, contributes to the stretch-induced response and to hypoxic tolerance.[89]

6 | NITRITE AS A BIOACTIVE NO SOURCE AND ENDOCRINE MEDIATOR

The first observation that endogenous NO circulates in plasma as S-nitrosothiol species[34] provided the rationale to consider these adducts as a NO reservoir by which the short-lived free gas can be preserved to mediate actions distal from its production site, thus eliciting endocrine effects. After these seminal data, it was found that exogenous NO, administered by inhalation[90] or intra-arterial application[91] is transported in the blood so that activation of downstream signalling cascades or formation of intermediates provides the gas of a longer half-life. Based on these premises, in 2003, Schechter and Gladwin[92] speculated about "NO as a hormone," and in 2006, the same authors proposed that circulating nitrite, being operative on targets which are distal from the production site, acts as a vascular endocrine NO reservoir.[93] Nitrite reduction to NO occurs through enzymatic and non-enzymatic pathways, including acidic disproportionation and reaction with endogenous nitrite reductases, such as Hb, Mb, neuroglobin, cytoglobin, xanthine oxidoreductase, eNOS and some mitochondrial enzymes.[94] These mechanisms, which have variable potency and functions at different PO_2 values, allow a nitrite-dependent NO production under hypoxic situations,

where NOS-catalysed NO formation may be compromised by a shortage of O_2 availability. In mammals, nitrite-derived NO contributes to hypoxic vasodilation, modulation of cellular respiration and cytoprotection during hypoxic/anoxic insults.[95] However, nitrite may also act as a signalling molecule on its own and regulates gene and protein expression.[96]

7 | NITRITE AS A CARDIOACTIVE AGENT

7.1 | Mammals

The mechanical performance of the mammalian heart is remarkably influenced by nitrite. On the Langendorff rat heart preparation, nitrite elicits concentration-dependent negative inotropic and lusitropic effects, decreasing left ventricular (LV) pressure and $LVdP/dt_{max}$ (indexes of contractility). Nitrite-dependent cardiodepression is mediated by the NO/sGC/PKG pathway but is unaffected by NOS inhibition, suggesting a NOS-independent mechanism.[97] In the same type of preparation, nitrite profoundly influences the preload-induced increases in LVP and $LVdP/dt_{max}$, acting as an intracardiac source of NO.[97] Noteworthy, nitrite positively affects myocardial relaxation, as shown by variations of the lusitropic index $LVdP/dt_{min}$ detected in Langendorff rat heart preparations exposed to the anion.[97]

7.2 | Fish

Data obtained on *A. anguilla* and on Antarctic Hb-less icefish *C. hamatus* indicate that also the fish heart performance is influenced by nitrite.[98] These teleost species are particularly suited for studying the nitrite/NO signalling due to their peculiar traits. The eurytherm and euryhaline eel experiences large fluctuations in environmental O_2 and is a champion of prolonged hypoxia and acidosis tolerance. In the eel, nitrite reduces the contractility of the isolated heart perfused under normoxic conditions, as shown by the decrement of stroke volume and stroke work. These effects depend on NOS activity, require the activation of the cGMP/PKG pathway and are accompanied by protein S-nitrosylation.[98] Contrary to the eel, the icefish is an extreme stenotherm endemic of the stable, icy and O_2-rich Antarctic waters. It lacks Hb and thus is deprived of a key NO homoeostatic protein responsible for NO scavenging and NO generation from nitrite.[98] In the icefish, contrary to the eel, and similar to the effect induced by NO,[98] nitrite induces a NOS-dependent increase in contractility. It has been proposed that the reduction of nitrite to NO occurs *via* cardiac Mb that, in the absence of Hb, represents the predominant form of nitrite reductase in the icefish.[98] A cardiac role of nitrite has been also proposed in the zebrafish, in which nitrite accumulation across the gills induces a relevant nitrite-to-NO conversion, increases Hb-NO levels and potentiates cardiovascular effects such as vasodilation and hypotension.[99] These observations suggest that like in mammals, also in poikilotherms, haem proteins play a central role in the nitrite/NO equilibrium. This is further supported by data in reptiles (*T. scripta*[100]) in which, in the presence of low O_2, Hb acts as a nitrite reductase.

7.3 | Amphibians

Physio-pharmacological evaluations on the isolated and in vitro perfused heart of the frog *R. esculenta* show that nitrite, acting as a physiological source of NO, depresses the ventricular contractile performance. Negative inotropic effect occurs *via* activation of the classic intracellular NO-dependent cascade as it is sensitive to NO scavenging and to cGMP and PKG inhibition.[98] Different from the basal cardiodepressive effect, in the frog heart, nitrite positively modulates the stretch-induced response. As shown in a comparative study carried out on the isolated and perfused eel, frog and rat heart, exogenous nitrite enhances stroke volume and stroke work in response to preload increases, similar to endogenous NO.[97] In all species, this effect is sensitive to NO scavenging by PTIO and persists after nitroxyl scavenging by NAC. This indicates that regardless of the evolutionary position and the type of morpho-functional arrangement, in the vertebrate heart NO acts as a nitrite-derived inotropic agent to modulate the preload-induced regulation of stroke volume. Of relevance, in the frog heart, application of the sGC inhibitor ODQ and the PKG antagonist KT5823 showed that nitrite involves a cGMP/PKG-dependent mechanism to enhance the Frank-Starling response. As the effects observed in the frog are similar to those elicited in eel and rat, they can be considered part of an evolutionary conserved mechanism of modulation of the stretch response activated by nitrite-derived NO. Interestingly, in frog, as in eel, hearts exposed to the Frank-Starling challenge in the presence of nitrite showed higher levels of S-nitrosylated proteins than those observed in the rat heart. This suggests that with respect to mammals, in poikilotherms, the role of this NO-dependent post-translational protein modification in transducing the effects of nitrite on the myocardial stretch response may be more important than in the mammalian heart.[97]

8 | CO

CO is produced in mammalian cells during haem catabolism by haem oxygenase (HO) enzymes, together with ferrous iron (Fe^{2+}) and biliverdin.[101] Three HO isoforms are

present in mammals: the inducible HO-1 (also known as heat shock protein 32 [Hsp32]) and the 2 constitutive isoforms, HO-2 and HO-3.[102] HO-1 is hyperexpressed in all tissues following many endogenous and exogenous stimuli, including haem derivates, NO and NO donors, hyperoxia, hypoxia, oxidative stress, AngII and glucose deprivation.[102] HO-2 is expressed in many tissues, including cerebral endothelial and smooth cells, and its activity is controlled by several endocrine modulators.[102] Interestingly, HO-2 represents a point of convergence for the NOS-NO system. In fact, as shown in mammalian retinal cells, the enzyme is upregulated by NOS inhibition.[102] The other constitutive CO-forming enzyme, HO-3, is considered a splice variant of HO-2, whose function is still unclear.[102] Different from the other gasotransmitters, under normoxia, CO is not metabolized and this allows the gas to have a long half-life of up to 3-7 h.[103]

Studies mainly carried out in mammals show that CO influences many physiological responses, acting on the gastrointestinal, renal, reproductive, nervous and immune systems.[102] Thanks to its control of carotid body activity, the gas is an important modulator of respiration.[104] In addition to these effects, CO acts on cardiovascular system by modulating blood pressure and angiogenesis and by inhibiting platelet aggregation.[103] It is generally considered a ubiquitous vasodilator,[105,106] although information on its vascular effects is heterogeneous. For example, it was observed that CO dilates cerebral arterioles of pig,[107] but not of rabbits and dogs;[106,108] it vasoconstricts rat *gracilis* muscle arterioles,[109,110] but does not affect canine and rabbit basilar and middle cerebral arteries,[108] and rat hepatic arteries.[111] CO vascular effects are elicited *via* recruitment of sGC,[112] Ca^{2+}-activated K^+ channels,[113] p38 mitogen-activated protein kinase (MAPK)[114] and NOS induction.[115]

Recent studies have extended to poikilotherms the role of CO as an endogenous physiological regulator. Teleost fish express both HO-1 and HO-2 and thus are able to endogenously generate CO. CO-forming enzymes were detected in cyprinid species, such as *C. auratus*,[116] *Cyprinus carpio*,[117] *Danio rerio*[118] and *Megalobrama amblycephala*,[119,120] and in the European sea bream *Dicentrarchus labrax* L.[121] Contrary to teleost, few data document the presence of CO-forming enzymes in amphibians.[122]

As in mammals, in poikilotherms, CO elicits important effects. Endogenous CO plays an inhibitory role in the control of breathing in larval and adult zebrafish,[123] as well as in goldfish in response to changes in water O_2.[124] At the same time, exposure to the CO donor, carbon monoxide-releasing molecules (CORM)-3 induces vasorelaxation of efferent branchial and celiacomesenteric arteries of the rainbow trout, and of the dorsal aorta of the sea lamprey *Petromyzon marinus*.[106] As non-specific HO inhibition elicited a dose-dependent increase in tension in trout efferent branchial arteries, this suggests a tonic control of vascular motility mediated by HO-generated gas. CO acts as a positive endogenous modulator of the sGC/cGMP signalling pathway in the neuromuscular junction of *Rana ridibunda*[125] and in the retina of salamander (*Ambystoma tigrinum*).[126] The same CO-activated sGC/cGMP signalling has been evidenced in reptiles (turtle[127]).

9 | CO AS A CARDIAC GASOTRANSMITTER

9.1 | Mammals

In the mammalian heart, CO is generated by HO-1 and HO-2 localized in atrial and ventricular myocytes. HO-1 expression is stimulated by stress conditions such as myocardial infarction,[128] hypoxia[129] and ischaemia/reperfusion (I/R).[130] In contrast, HO-2 is constitutively expressed in atrium, ventricle and intracardiac neurones.[131,132] As observed in the in vivo dog heart, the enzyme is activated during myocardial ischaemia. The following CO generation stimulates a cGMP-dependent cascade that contributes to vasodilate the coronary microcirculation.[133] Recently, it has been reported in perfused heart, and isolated cardiomyocytes of Sprague Dawley rats, that increased expression of HO-2 and of its activator biliverdin reductase, promotes cardiomyocyte survival, protecting against isoproterenol-dependent cardiotoxicity.[134] Also CO generation by exogenous CORMs confers protection against I/R injuries, both in vivo and in vitro.[135] In in vivo mice, pre-treatment with CORM-3 activates a cardioprotective cascade *via* the upregulation of anti-apoptotic and cytoprotective mechanisms that recruit the transcription factors NF-κB, STAT1/3 and Nrf2.[136] At the same time, in isolated rat hearts, exogenous CO protects from I/R injury *via* K_{ATP} channels, p38 MAPK-β, PKC and PI3K.[137,138]

9.2 | Fish and amphibians

Few stimulating data document the presence of a HO/CO system in the poikilotherm heart. Both HO-1 and HO-2 have been identified in teleost and amphibians, revealing a high sequence conservation with respect to mammals. HO-1 enzyme characterized in zebrafish shares 50% identity with the sequence detected in mammals and birds (for references, see ref. 11), 86% with the protein found in goldfish[116] and 60% with that identified in *Xenopus*.[122] In the zebrafish heart, it is expressed under basal conditions. Consistent with the characteristic inducibility of the enzyme, expression increases after hypoxia/reoxygenation, exceeding that of other hypoxia-sensitive markers, such as VEGF and erythropoietin. HO-1 increased expression is associated

with cardiac protection.[139] In the developing zebrafish heart, the enzyme is located on presumptive pacemaker cells, and this suggested a role on setting heart rate.[118] Recently, HO-2 was detected in the heart of the blunt snout bream *M. amblycephala*, where the enzyme is present in 2 isoforms, HO-2a and HO-2b, generated by gene duplication. These are differently regulated by hypoxia and follow a developmental pattern. In the adult, hypoxia upregulates HO-2a and downregulates HO-2b mRNA expression. In contrast, in the embryo, the expression of both HO-2a and HO-2b mRNA is enhanced by hypoxic stress.[120]

Currently, the physiological role of CO in the heart of fish and amphibians has been only partially explored. In larval and adult rainbow trout, in vivo exposure to CO is accompanied by a positive chronotropic response.[140] A similar response was found in both larval trout[140] and HO-1-expressing zebrafish larvae exposed to hypoxia.[118] As in mammals, this effect has been correlated with a modulation of the sympathetic nervous activity by the HO-1/CO system (for references, see ref. 118). In contrast, zebrafish larvae lacking HO-1 exhibited a significant higher heart rate under normoxia, but not hypoxia. This effect, rescued by exogenous CORM-derived CO, was insensitive to adrenaline, suggesting that under normoxia the cardiac effects of endogenous CO are independent from the autonomic regulation.[118] The finding that in zebrafish larvae gasotransmitters are involved in cardiac function modulation also in relation to oxygen availability should be regarded with interest for exploring mechanisms able to improve mammalian myocardium resistance to oxygen deprivation (ie during ischaemia).

In amphibians, to the best of our knowledge, only 1 study carried out in clawed frog *X. laevis* analysed the influence elicited by CO on the cardiovascular system.[141] Data show that during the early development, exposure to CO does not affect oxygen consumption, lactate concentration and individual mass, indicating that limiting blood O_2 transport does not impair metabolism and the cardio/respiratory function. Moreover, CO exposure increases cardiac output and stroke volume, but not heart rate, a response similar to that found in adult fish and reptiles, in which hypoxia increases cardiac output mainly as a result of an increase in stroke volume.[141]

10 | H_2S

H_2S, long considered a toxic pollutant, is now accepted as an endogenous mediator of important processes. It is involved in neurotransmission, neuroprotection, insulin release, inflammation, vessel motility and cardioprotection.[8] In mammals, it is produced *via* the cysteine metabolic enzymes CBS, CSE and 3MST coupled with cysteine aminotransferase (CAT).[7] CBS predominates in the brain, while CSE is the major H_2S-producing enzyme in the cardiovascular system, in which it is expressed in vascular smooth muscle,[142] endothelium[143] and myocardium.[144]

L-cysteine represents a major substrate for H_2S production; however, also D-cysteine has been proposed as a substrate for D-amino acid oxidase (DAO) catalysis to generate H_2S.[145,146] The main H_2S-dependent signalling mechanism is S-sulfhydration, that is the addition of a -SH group to reactive cysteine residues on target proteins, analogous to NO-induced protein S-nitrosylation.[147] Additional mechanisms are represented by direct reaction with reactive oxygen, nitrogen species and metal centres.[148] Major targets of H_2S are membrane intracellular ion transport systems. They include ATP-dependent K^+ channels,[149] Ca^{2+}-activated K^+ channels, T- and L-type Ca^{2+} channels, sodium/calcium exchangers, chloride channels, transient receptor potential channels, β-ARs and N-methyl-D-aspartate receptors.[150,151]

11 | H_2S AS A CARDIAC GASOTRANSMITTER

11.1 | Mammals

Ongoing studies in mammals are providing interesting information on the cardiovascular modulation induced by H_2S. One of the first "physiological" effects attributed to H_2S is the vasodilation of systemic vessels (rat thoracic aorta and portal vein).[142] This H_2S-dependent vascular smooth muscle relaxation is attributed to K_{ATP} channel opening[152] and S-sulfhydration of the sulfonylurea receptor 1 (SUR1) subunit of the channel.[153] The H_2S-dependent vasodilation of systemic vessels is similar to the response elicited by hypoxia. Like CO and NO, H_2S acts as a very sensitive oxygen sensor. As shown in cells from the carotid body, this action seems to occur by regulating O_2 sensing *via* ion channels (eg maxiK) modulation.[154]

At cardiac level, intravenous H_2S administration does not modify heart rate, but reduces blood pressure in rat.[155] Differently, in rabbit, H_2S generated by NaHS reduces heart rate by inhibiting sinoatrial cells. This effect is blocked by glibenclamide, thus confirming the involvement of K_{ATP} channels.[156] H_2S also blunts the contractile performance of cardiomyocytes *via* a modulation of K_{ATP} channels, L-type Ca^{2+} channels and the cAMP/PKA pathway.[144,157,158] Interestingly, during I/R injury, H_2S plays a protective role by inducing hypometabolism associated with anti-inflammatory, anti-apoptotic and antioxidant effects.[159–161] Protection also occurs *via* modulation of K_{ATP} channels opening, inhibition of mitochondrial respiration and the preservation of mitochondrial membrane integrity.[162]

11.2 | Fish and amphibians

The cardiovascular activity of H_2S in poikilotherm vertebrates is weakly documented. The available data mainly concern its vasoactive properties that represent a conserved functional trait of this gasotransmitters, being present from fish[9,163,164] to reptiles.[165] In the classes so far examined, the gas elicits in vivo and in vitro vasoconstrictory, vasodilatory or multiphasic responses. These are species- and vessel-specific and may depend on either duration or dose of exposure.[9,163,164] For example, in fish, H_2S produces an endothelium-independent monophasic contraction of the dorsal aorta of sea lamprey and hagfish, a monophasic relaxation of the shark dorsal aorta,[9] and a multiphasic (ie relaxation-contraction-relaxation) modulation of the trout efferent branchial artery.[163] In the last case, relaxation depends on K_{ATP} channels and/or prostanoids of endothelial origin, while the contractile phase partially depends on extracellular Ca^{2+}.[163] Also in amphibians the gas modulates the vascular performance, eliciting dose-dependent contraction in marine toad aorta, and a multiphasic effect (ie contraction-relaxation-contraction) on pulmonary arteries.[163] Of note, as in mammals, an extensive number of studies encompassing all classes of vertebrates, and irrespective of species or vascular beds, show that H_2S exhibits identical responses of hypoxia (for references, see ref. 166).

Contrary to the growing evidence on the vascular role of H_2S, the cardiac effects of the gas in fish and amphibians remain almost unexplored and limited to its exogenous effects in vivo. In trout, intrabuccal H_2S injection is accompanied by bradycardia.[167] As the gas is not present in plasma,[168] it can be excluded that it acts as a circulating signalling molecule. Interestingly, under hypoxic conditions, the trout myocardium generates H_2S in the presence of cysteine, suggesting that like in mammals, also in teleost, it plays a cardioprotective role.[168] Recently, Wu et al[169] reported that H_2S influences the teleost heart during development. By exposing zebrafish embryos to low H_2S (0.02, 0.05 and 0.08 mmol/L) obtained by exogenous Na_2S administration, they observed an increased heart rate and development velocity. The positive chronotropic effect was similar to that observed in rat in the presence of heart failure as a consequence of microinjection of a H_2S donor GYY4137 in the paraventricular nucleus[170] and was attributed to the cardioprotective abilities of the gas.[171] In contrast, exposure to high H_2S concentrations (0.10 and 0.20 mmol/L) resulted in a decreased heart rate. This effect, as persisted after embryos transfer to reconstituted water, was considered deleterious for the developing zebrafish heart.[170]

Also the amphibian heart is sensitive to H_2S. On isolated ventricular strips of the frog *R. ridibunda*, H_2S reduces contractility.[172] A similar response has been observed on the isolated and in vitro perfused working heart of *R. esculenta* after exposure to exogenous H_2S generated by NaHS.[173] The effect is dose-dependent (10^{-12}-10^{-7} mol/L) and results in a decrement of about 40% of stroke volume and stroke work at the highest concentrations. Because of the absence of coronary vasculature, this effect has been attributed to an action of the gas on the myocardium, via the involvement of the EE. As H_2S-induced negative inotropism is counteracted by glibenclamide, it was proposed that the mechanism of action recruited by the gas involves K_{ATP} channels. Moreover, NaHS treatment increases cytosolic protein S-sulfhydration, suggesting that in frog, as in mammals, this post-translational modification represents an additional mechanism for H_2S-dependent control of cardiac function.[173] The fact that it is present in both amphibians and mammals indicates its evolutionary importance in the context of H_2S-dependent modulation of heart performance.

Although ongoing studies are providing interesting information on the role of H_2S in the cardiac physiology of vertebrates, many aspects remain to be explored. A good opportunity to this purpose is the use of natural animal models that survive to high H_2S levels thanks to many strategies that include (i) modifications of the integumentary and respiratory surfaces, both directly exposed to H_2S; (ii) potentiation of the mechanisms responsible for H_2S elimination; and (iii) reduced sensitivity of H_2S toxicity targets. Examples of adaptation to H_2S-rich environments are the teleost belonging to the genus *Poecilia* (ie *P. mexicana, P. sulphuraria, P. thermalis*). They independently colonized 3 Mexican springs characterized by toxic H_2S concentrations, show peculiar phenotypic traits represented by enlarged heads and an increased branchial surface area that facilitates oxygen acquisition. They also are characterized by physiological and biochemical adaptations which include a decreased susceptibility of cytochrome c oxidase complex to H_2S which reduces the damages induced by sulphide toxicity.[174–176]

12 | CROSSTALK AMONG GASOTRANSMITTERS

An open and promising field of research, mainly developed in mammals, regards the functional and biomolecular interactions among gasotransmitters. Many regulatory roles and molecular targets are common to NO, CO and H_2S. For all of them, reactivity is controlled by maintaining low steady state levels (from nanomolar to picomolar range), thanks to an active balance between generation and rapid metabolic consumption. In the case of NO and H_2S, both use an amino acid for enzymatic production (L-arginine for NOS

and L-cysteine for CSE and CBS). All gases induce vasodilation, apoptosis and anti-inflammatory mechanisms and inhibit oxidative phosphorylation with both NO and H_2S acting on cytochrome oxidase. At the same time, they interact with Hb, so that the binding of 1 gasotransmitter to the protein affects the binding of the others. NO and CO are both able to interact with the haem group of sGC and induce cGMP formation, but NO has a higher affinity with respect to CO. Interestingly, CO does not stimulate sGC in the presence of low NO levels (for references, see ref. 4). H_2S interacts with Hb, Mb and cytochrome oxidase but not with the haem group of sGC. At the same time, H_2S influences downstream the cGMP-dependent cascade by inhibiting PDE-dependent degradation of the cyclic nucleotide.[177,178]

Interestingly, the 3 gasotransmitters reciprocally modulate their production. For example, in a model of pressure-overload murine heart failure, H_2S induces phosphorylation and activation of eNOS, thus increasing NO generation.[179] At the same time, NO donors increase CSE expression in aortic smooth muscle cells.[152] Another point of interaction is the modification induced by NO and H_2S on protein sulfhydryl groups. This is the case of NO-dependent S-nitrosylation and H_2S-dependent S-sulfhydration. Intriguingly, these modifications are often responsible of opposing effects. S-sulfhydration increases activity of the modified proteins,[180] while S-nitrosylation often reduces them.[181] Because of the specific kinetics of these reactions, it was proposed that S-sulfhydration inhibits the subsequent S-nitrosylation of the same protein and this represents a powerful mechanism of reciprocal control, crucial for the homoeostatic equilibrium among gasotransmitters.[180]

Also CO and H_2S appear to reciprocally interact, although the limited information on this aspect is still under debate. For example, a cycle in which H_2S/CSE pathway inhibits CO generation and the CO/HO system inhibits H_2S production appears to characterize the crosstalk between these 2 gasotransmitters.[182] The physiological significance of these relationships is unresolved. As suggested by observations in carotid bodies, CO-H_2S interaction may importantly contribute to oxygen sensing. Under normoxia, the inhibitory influence of CO on CSE suppresses H_2S generation, whereas during hypoxia, a reduced CO production relieves the inhibition on CSE, leading to elevated H_2S levels, which contribute to sensory excitation, and this may be of relevance for matching the respiratory requirement and the cardiovascular performance.[154]

Contrary to the still growing studies on mammals, few researches in poikilotherms attempted to describe the crosstalk between gasotransmitters, particularly in relation to their influence on the cardiac function. A functional indication concerns a crosstalk between NO and H_2S as highlighted by physio-pharmacological and biomolecular observations on the frog heart,[173] in which endogenous NO generation is required for H_2S-induced effects. In fact, NOS inhibition by L-NMMA reduces the cardiosuppression induced by NaHS. In the frog heart, H_2S utilizes the cGMP/PKG pathway to elicit depressant effects on contractility and this represents a convergent mechanism for the 2 gases, similar to that documented in mammals, as well as in reptiles.[126] Of note, H_2S and the NO systems interact upstream NOS activation, as H_2S stimulates the Akt-dependent eNOS phosphorylation. Interestingly, in the avascular frog heart, the functional damage of the major cardiac NO-generating tissue, that is the EE, abolishes the NaHS-induced negative inotropism and prevents the increase in p-eNOS expression induced by H_2S (Figure 3).

13 | GASOTRANSMITTERS AND CARDIAC PLASTICITY: THE FISH NOS/NO SYSTEM AS AN EXAMPLE

Among non-mammalian vertebrates, fish are champions of adaptive cardiac flexibility. The about 20 000 extant teleost species show an extraordinary interspecific morpho-functional variability of the heart, as a result of an evolutionary adaptation to different challenges that include food and oxygen deprivation, sexual maturation, exercise training and rearing under aquaculture conditions, and temperature challenges (see, eg, refs 183–186). Recent experimental evidence suggests that in fish, gasotransmitters play a role in preserving cardiac function under different conditions, as in the case of changes in temperature and oxygen availability and exposure to humoral stressors. This hypothesis is mainly based on studies that document the influences elicited on the heart by the NOS/NO system. For example, using the isolated working heart of *A. anguilla*, Amelio et al[187] reported a functional relationship between temperature challenges and the NO-dependent modulation of the Frank-Starling response. While in the perfused heart of long-term temperature acclimated fish (ie spring animals perfused at 20°C and winter animals perfused at 10°C) inhibition of the NOS-dependent NO production significantly reduced the response to preload increases, under thermal shock (spring animals perfused at 10 or 15°C and winter animals perfused at 15 or 20°C), NOS inhibition is without effect.[187] The decreased NOS expression observed in the ventricle of eels exposed to thermal shock confirms that a modulation of the NOS/NO system is crucial for enabling the fish heart to respond to temperature challenges, as well as for maintaining homoeostasis at physiological temperatures.

A role for the intracardiac NOS/NO system in the response to hypoxia/anoxia has been proposed in goldfish (*C. auratus*) and crucian carp (*C. carassius*).[31,188] It is

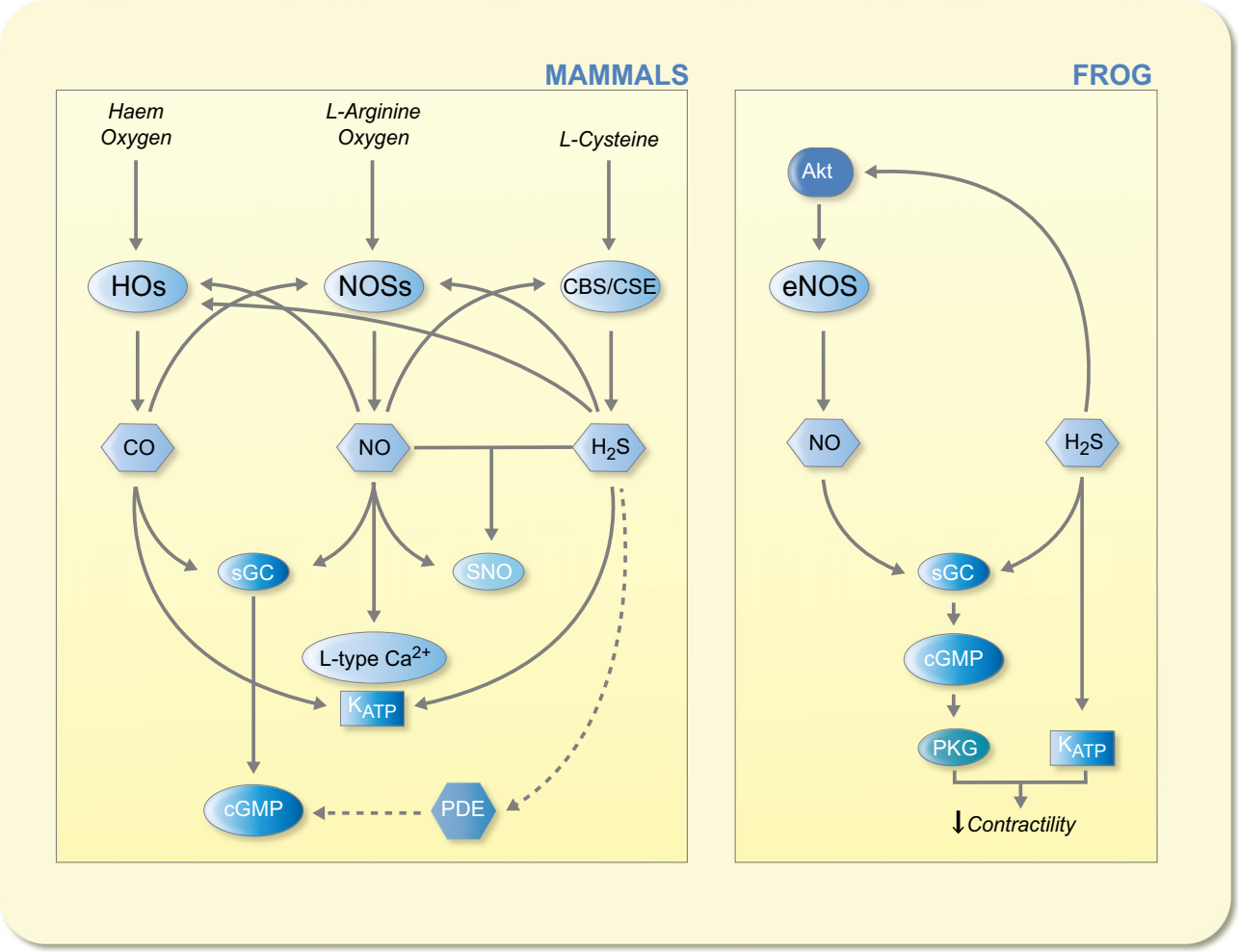

FIGURE 3 Simplified scheme showing the biosynthetic pathways for gasotransmitters generation and their intracellular targets in mammals. The convergence of CO and NO on sGC and the inhibitory relationship between H_2S and the NOS system are illustrated. The involvement of Akt in the crosstalk between NO and H_2S in the frog heart is also reported. For details and references, see the text

known that a reduced oxygen availability compromises NOS-mediated NO generation. Under these conditions, an increased cardiac NOS expression, or a nitrite-to-NO conversion, possibly contributes to stabilize myocardial NO levels.[31] In line with these observations, Imbrogno et al[45] showed that the isolated and perfused goldfish heart, working under physiological conditions and exposed to both normoxia and hypoxia, is under a tonic NO-cGMP modulation that enhances the Frank-Starling response. In the presence of reduced oxygen availability, cardiac NOS expression is increased, suggesting that an elevation of NO production contributes to adjust the heart performance during hypoxic challenges. This may be crucial for maintaining the functional and metabolic interactions between organs and tissues that are required for the hypoxia tolerance of the organism. Interestingly, in the anoxic crucian carp, the sustained cardiac output, the reduced peripheral vascular resistance and the conserved autonomic control are critical for preventing ethanol accumulation and intoxication in tissues.[189]

As shown in the cardiac tissue of *C. carassius,* also anoxia is accompanied by an increase in nitrite and NO levels, and this has been considered protective for the cardiac tissue against potential reoxygenation damage.[31] Using a physio-pharmacological approach associated with immunolocalization and biomolecular techniques on the eel (*A. anguilla*) heart, Imbrogno et al[21] described a protective role of NO also towards excessive excitatory humoral stimuli. They found that chronic administration of AngII improves the performance of the eel heart, enhancing its ability to sustain increased afterload. This AngII-mediated effect, indicative of a morpho-dynamic remodelling of the heart, is accompanied by an increase in "p-eNOS-like" isoform expression, and thus of NO generation. The authors speculate that the increased NO bioavailability observed in AngII-stimulated eel hearts may serve to counterbalance

the effects of growth-promoting signalling, thus contributing to cardioprotection.[21,50]

14 | CONCLUSIONS

Since the discovery of the physiological properties of NO, many advances have been made for understating its role, as well as that of CO and H_2S, in cardiovascular modulation. In addition to the impressive work aimed to explore their cardiovascular properties in mammals, recent research unrevealed the primordial cardiac function of all 3 gasotransmitters in non-mammalian heart models, locating them in a wider biological framework than so far envisioned.

There is now robust evidence that also in poikilotherms, these molecules coordinate the capacity of the heart to sense, translate and respond to short-, medium- and long-term physiological requirements induced by variations of both internal and external milieus. This is a fundamental task for the continuously beating heart, also in relation to the needs imposed by often severe ontogenetic and phylogenetic limitations.

The large spectrum of morpho-functional arrangement and molecular traits that characterize the cardiac design of the numerous species of fish and amphibians prevents the formulation of general categorizations concerning the role of NO, CO and H_2S as cardiac modulators in non-mammalian vertebrates. However, the presence of aspects of unity in terms of gasotransmitters effects, intracellular cascades, etc., makes the information so far available in poikilotherms a useful conceptual background in which to locate studies aimed not only to improve basic knowledge on these fascinating animals, but also to dissect aspects which are difficult to explore in more complex cardiac designs, as in mammals.

CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

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RESEARCH ARTICLE

The roles of tissue nitrate reductase activity and myoglobin in securing nitric oxide availability in deeply hypoxic crucian carp

Marie N. Hansen[1], Jon O. Lundberg[2], Mariacristina Filice[3,4], Angela Fago[4], Nanna M. G. Christensen[1] and Frank B. Jensen[1,*]

ABSTRACT

In mammals, treatment with low doses of nitrite has a cytoprotective effect in ischemia/reperfusion events, as a result of nitric oxide formation and *S*-nitrosation of proteins. Interestingly, anoxia-tolerant lower vertebrates possess an intrinsic ability to increase intracellular nitrite concentration during anoxia in tissues with high myoglobin and mitochondria content, such as the heart. Here, we tested the hypothesis that red and white skeletal muscles develop different nitrite levels in crucian carp exposed to deep hypoxia and assessed whether this correlates with myoglobin concentration. We also tested whether liver, muscle and heart tissue possess nitrate reductase activity that supplies nitrite to the tissues during severe hypoxia. Crucian carp exposed to deep hypoxia ($1<P_{O_2}<3$ mmHg) for 1 day increased nitrite in red musculature to more than double the value in normoxic fish, while nitrite was unchanged in white musculature. There was a highly significant positive correlation between tissue concentrations of nitrite and nitros(yl)ated compounds. Myoglobin levels were 7 times higher in red than in white musculature, but there was no clear correlation between nitrite and myoglobin levels. Finally, we found a low but significant nitrate reductase activity in liver and white muscle, but not in cardiomyocytes. Nitrate reduction was inhibited by allopurinol, showing that it was partly catalyzed by xanthine oxidoreductase.

KEY WORDS: Ethanol, Hypoxia, Nitrate reduction, Nitric oxide, Nitrite, Red muscle

INTRODUCTION

Nitric oxide (NO) is a vital signaling molecule that exerts its physiological effects by reversible binding/reacting with hemes, thiols or amines, forming iron-nitrosyl (FeNO), *S*-nitroso (SNO) and *N*-nitroso (NNO) compounds (Hill et al., 2010). Furthermore, NO is short-lived and excess NO is rapidly oxidized to nitrite and nitrate. Under normoxic conditions, NO is produced from the reaction of L-arginine with molecular oxygen, catalyzed by nitric oxide synthase (NOS) enzymes, and this reaction is vulnerable to hypoxia because of the requirement for O_2 (Moncada, 1993; Bryan, 2006; Lundberg et al., 2008). However, it has been established that both nitrite and nitrate can be reduced back to NO, which provides an alternative pathway for NO generation under hypoxia (Gladwin et al., 2005; Lundberg et al., 2009; van Faassen et al., 2009). Accordingly, NO is generated by different mechanisms depending on oxygen levels and also tissue type, pH and redox status (Gladwin et al., 2005; Feelisch et al., 2008; Hill et al., 2010). The preservation of NO availability by nitrite reduction is indeed important in hypoxia, where it contributes to hypoxic vasodilation and cytoprotection (Cosby et al., 2003; Shiva and Gladwin, 2009). Several studies have documented that administration of nitrite can reduce cell death and infarct size after ischemia/reperfusion in heart and liver tissues of mammals (Webb et al., 2004; Duranski et al., 2005; Hendgen-Cotta et al., 2008; Shiva and Gladwin, 2009). This relates to the binding of nitrite-derived NO to complex IV of the mitochondrial respiratory chain, which reduces respiration rate and extends the O_2 gradient, as well as *S*-nitrosation of complex I, which limits generation of reactive oxygen species (ROS) at the onset of reoxygenation (Shiva et al., 2007; Murillo et al., 2011; Chouchani et al., 2013). Interestingly, anoxia-tolerant vertebrates (crucian carp and freshwater slider turtles) seem to naturally exploit these mechanisms by elevating nitrite and nitros(yl)lated compounds in, for example, the heart during deep hypoxia and anoxia (Sandvik et al., 2012; Jensen et al., 2014; Hansen et al., 2016).

We have previously shown that goldfish and crucian carp maintain tissue nitrite concentration during hypoxia and increase it in the heart during deep hypoxia and anoxia (Hansen and Jensen, 2010; Sandvik et al., 2012; Hansen et al., 2016). This occurs in the face of a decrease in extracellular nitrite concentration. We suggested that nitrite is transferred from the extracellular to the intracellular space and that this could be facilitated by intracellular binding of nitrite to proteins, which would keep the cytosolic concentration of free nitrite low and permit inward diffusion (Hansen and Jensen, 2010). Because anoxia increases tissue nitrite in the heart of crucian carp and red-eared slider turtles, as well as in red pectoral muscle of turtles, but not white muscle of crucian carp, we hypothesized that myoglobin (Mb) or mitochondria, both present at high levels in heart and red musculature, play a role (Jensen et al., 2014). Specifically we suggested that the increased binding of negatively charged nitrite during anoxia could be explained by a progressively more positively charged Mb due to H^+ buffering caused by anoxia-induced acidosis (Jensen et al., 2014). One aim of the present study was to measure nitrite levels in red and white muscle of normoxic and deeply hypoxic crucian carp to validate a difference in nitrite levels between the two muscle types within the same species. Further, we measured Mb concentrations in red and white muscle to test for a possible correlation between tissue nitrite and Mb levels.

As mentioned above, extracellular nitrite is shifted into tissues of hypoxic and anoxic fish. But the extracellular pool of nitrite is sparse, and nitrite needs to be supplemented from other sources to maintain or increase tissue nitrite concentration during long-term

[1]Department of Biology, University of Southern Denmark, Odense M DK-5230, Denmark. [2]Department of Physiology and Pharmacology, Karolinska Institute, Stockholm SE-17177, Sweden. [3]Department of Biology, Ecology and Earth Sciences, University of Calabria, 87036 Arcavacata di Rende (CS), Italy. [4]Zoophysiology, Department of Bioscience, Aarhus University, Aarhus C DK-8000, Denmark.

*Author for correspondence (fbj@biology.sdu.dk)

F.B.J., 0000-0001-6148-6663

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hypoxia. We recently showed that crucian carp can utilize ambient nitrite by taking it up across the gills and transporting it via the blood to tissues, where it enters cells, notably in hypoxia (Hansen et al., 2016). Another potential source of tissue nitrite is tissue nitrate. Mammalian tissues have been shown to possess nitrate reductase activity (mediated by xanthine oxidoreductase and possibly other proteins) that can reduce nitrate to nitrite, and this is most prominent during hypoxic/anoxic conditions (Jansson et al., 2008; Huang et al., 2010; Piknova et al., 2015). The existence of tissue nitrate reductase activity in fish remains to be established, but it could be a valuable additional source of nitrite (and thus NO) in crucian carp, when they experience long-term hypoxia and anoxia in ice-covered ponds during winter (Vornanen et al., 2009). For this reason, a major aim of the present study was to investigate nitrate reductase activity in selected tissues from normoxic and deeply hypoxic crucian carp. The liver was examined because of its relatively high nitrate reductase activity in mammals (Jansson et al., 2008; Piknova et al., 2015). The heart was tested for nitrate reductase activity because of the distinctive increase in nitrite concentration in this tissue during anoxia and deep hypoxia (Sandvik et al., 2012; Hansen et al., 2016). Finally, white skeletal muscle was examined because it constitutes some 50% of the fish mass, and because it has a unique role in anoxic crucian carp in converting lactate to ethanol (for subsequent excretion across the gills), thereby limiting acidosis during anaerobic metabolism (Shoubridge and Hochachka, 1980; Johnston and Bernard, 1983; Vornanen et al., 2009). To ascertain ethanol production in deep hypoxia, we measured ethanol concentration in muscle and plasma.

MATERIALS AND METHODS

Animals, treatment and sampling

Crucian carp, *Carassius carassius* (Linnaeus 1758), of mixed sex, weighing 45.4±1.85 g (mean±s.e.m., N=42) were caught in a local pond (Langsted, Funen, Denmark) in July and transferred to two 200 l holding tanks, where pond water was gradually changed to experimental water (Odense tap water mixed with demineralized water in a 1:4 ratio). The fish stayed in the tanks for 17 days and were fed with commercial trout pellets (Inicio, Biomar, Denmark), while being acclimated to 15°C and a 12 h:12 h light:dark cycle in normoxic (P_{O_2}>140 mmHg) water. Normoxia was obtained by bubbling air, and water was exchanged daily.

The fish were subsequently moved to four normoxic experimental aquaria (100 l, with 10–11 fish in each) for 5 days without feeding; 60 l of water was exchanged twice daily. Two aquaria were maintained normoxic (P_{O_2}>140 mmHg) for one additional day, while the two other aquaria were bubbled with N_2 for 1 day to expose the fish to deep hypoxia (1<P_{O_2}<3 mmHg). The water surface was covered with expanded polystyrene, and deep hypoxia was reached within 4 h. Water P_{O_2} was measured using an optical Hach Lange optode (HQ 40d, Loveland, CO, USA). Water nitrite concentration stayed below 0.5 µmol l^{-1} and water Cl^- concentration was 260 µmol l^{-1}.

Fish were caught individually and anesthetized in 2‰ MS222 (ethyl-3-aminobenzoate methanesulfonate) dissolved in experimental water. The fish were weighed and blood was taken from the caudal vessels, after which tissues were dissected out in the following order: heart, liver and muscle. The muscle was divided into red muscle (primarily slow oxidative fibers) and white muscle (primarily fast glycolytic fibers). The red muscle (musculus lateralis superficialis trunci) is situated in a thin layer underneath the skin along the lateral line of the fish (Hamoir, 1953). Blood was centrifuged (2 min, 8000 ***g***, 15°C) and plasma was separated. Tissues were rinsed in phosphate-buffered saline [50 mmol l^{-1} phosphate buffer pH 7.8; 85 mmol l^{-1} NaCl; 2.4 mmol l^{-1} KCl; 10 mmol l^{-1} *N*-ethylmaleimide (NEM); 0.1 mmol l^{-1} diethylenetriaminepentaacetic acid (DTPA)], dried on tissue paper and weighed. All samples were snap frozen in liquid nitrogen and stored at −80°C until further analysis.

The procedures and experiments were conducted in accordance with Danish laws on animal experimentation. All chemicals were purchased from Sigma-Aldrich (Steinheim, Germany).

Nitrate reduction and determination of NO metabolites

The method for determination of tissue nitrate reductase activity was mostly conducted as previously described (Jansson et al., 2008). In short, subsamples of tissues weighing between 19 and 200 mg (depending on the tissue) were thawed and homogenized in 3 times their weight of phosphate buffer (100 mmol l^{-1}, pH 7.0) and 1 vol % protease inhibitor (P2714, Sigma-Aldrich). Heart and liver tissues were homogenized using zirconium oxide beads (ZROB05, 5 mm, Next Advance, NY, USA) in a Bullet Blender Blue (Next Advance) and muscle tissue was homogenized with a tissue grinder (Struers, Heidoloph, Denmark). Homogenates were centrifuged (10 min, 500 ***g***, 4°C), and supernatants were decanted and divided into subsamples that were snap frozen, or used for determination of protein concentration, using the BioRad (München, Germany) protein assay based on Bradfords assay, with bovine serum albumin as standard (Bradford, 1976). Final concentrations of protein (7 mg ml^{-1}), cofactors (1 mmol l^{-1} NADPH, 500 µmol l^{-1} NADH, 500 µmol l^{-1} NAD^+, 500 µmol l^{-1} GSH) and nitrate (300 µmol l^{-1}) were achieved by mixing appropriate amounts of buffer (250 mmol l^{-1} sucrose, 10 mmol l^{-1} Tris-HCl, pH 7.0), homogenate, cofactor mix and nitrate in the given order. The volume of buffer was adjusted to fit the final volume in incubations with or without nitrate. Immediately after mixing, one aliquot was used to determine NO metabolites at time zero and the other subsample was subsequently bubbled with pure nitrogen for 2 min (heart and liver) or 7 min (muscle), sealed with Parafilm® and incubated for 5 h at 25°C before a further determination of NO metabolites. To ascertain a persistent N_2 atmosphere, we also performed experiments with liver homogenates in shaking Eschweiler (Kiel, Germany) tonometers receiving a continuous flow of humidified N_2. The role of xanthine oxidoreductase (XOR) in nitrate reduction was examined by adding the XOR inhibitor allopurinol at 2 mmol l^{-1}. Nitrite and nitros(yl)ated compounds were determined by chemiluminescence (NO analyzers: Model CLD 77 AM, Eco Physics, Duernten, Switzerland; and Model 280i, Sievers, Boulder, CO, USA), as previously described (Yang et al., 2003; Hansen and Jensen, 2010). Nitrate was assessed with a vanadium chloride assay, and nitrite and nitros(yl)ation compounds (SNO+FeNO+NNO) were determined in a triiodide assay. To distinguish between nitrite and [SNO]+[FeNO]+[NNO], the samples were treated with sulfanilamide (Hansen and Jensen, 2010). NO-metabolites were calculated as absolute concentrations in µmol l^{-1} assuming a tissue density of 1 kg l^{-1}. Protein determinations in liver, muscle and heart did not differ between normoxic and deeply hypoxic fish, indicating that hypoxia did not cause a significant water shift and hence did not influence NO metabolite concentrations.

Muscle Mb concentration

Mb concentration was determined spectrophotometrically using the method developed by Reynafarje (1963) with small modifications (Helbo and Fago, 2012). White muscle (~200 mg) and red muscle

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(~100 mg) were homogenized in 4.25 and 9.25 times their mass of hypotonic buffer (40 mmol l^{-1} $KHPO_4$, pH 6.60), respectively. We used a knife homogenizer (ultra-turrax T25, IKA-labortechnic, Staufen, Germany) on ice, 3 times 30 s with a 30 s break in between, to avoid heating up the homogenates. After centrifugation (50 min, 15,000 ***g***, 4°C), homogenates containing Mb were equilibrated with CO for 3 min and ~0.001 g dithionite was added to reduce any ferric heme (Helbo and Fago, 2012). Absorbance spectra were collected from 700 to 400 nm, and Mb concentration (mg g^{-1} wet mass) was determined from the difference in absorbance at 538 and 568 nm [which is zero for carboxyhemoglobin (HbCO) but not for carboxymyoglobin (MbCO)], using reported extinction coefficients for the corresponding dilution (Reynafarje, 1963).

Ethanol

Ethanol was determined in plasma and white muscle homogenates using a commercial ethanol assay kit (MAK076, Sigma-Aldrich) and a SpectraMax Plus Microplate Reader (Molecular Devices, Sunnyvale, CA, USA).

Statistics and data processing

Graphing and statistical analyses were performed in Prism 5.0 (GraphPad Software, San Diego, CA, USA) and Origin 8.5 (OriginLab Corporation, Northampton, MA, USA). Results are presented as means±s.e.m. and statistical differences between means were evaluated using unpaired *t*-test, one-way analysis of variance (ANOVA) with Bonferroni *post hoc* test or two-way ANOVA, as appropriate. Furthermore, linear regression and one-sample *t*-test (to compare means with zero) were used. We applied a significance level of $P<0.05$ and data that did not fulfil the assumption of equal variances (Bartlett's test) were log-transformed prior to analysis.

RESULTS

Crucian carp exposed for 1 day to deep hypoxia showed slightly lower plasma nitrite concentration (Fig. 1A) and plasma [SNO+FeNO+NNO] (Fig. 1B) compared with normoxic fish. Plasma nitrate concentration (Fig. 1C) did not differ between normoxic and deeply hypoxic groups.

In red skeletal muscle, nitrite concentration increased to more than double the normoxic value after exposure for 1 day to deep hypoxia, whereas it stayed constant in white skeletal muscle at a value that was similar to that in normoxic red muscle (Fig. 2A). The rise in red muscle nitrite concentration during deep hypoxia was paralleled by a significant increase in the concentration of nitros(yl) ated compounds (SNO+FeNO+NNO; Fig. 2B). In white muscle, [SNO+FeNO+NNO] did not change (Fig. 2B). A plot of all individual muscle [SNO+FeNO+NNO] values against corresponding nitrite concentrations revealed a highly significant (R^2=0.811) linear increase in [SNO+FeNO+NNO] with increasing nitrite concentration (Fig. 3). Tissue nitrate concentration was slightly higher in white than in red muscle but only decreased non-significantly during deep hypoxia (Fig. 2C).

The level of Mb was significantly higher in red muscle than in white muscle but was unaffected by the level of ambient oxygen (Fig. 4). Hence, red muscle Mb was 3.68±0.369 mg g^{-1} (corresponding to 0.22 mmol l^{-1}) in normoxic fish and 2.98±0.362 mg g^{-1} in deeply hypoxic fish, which is 7–8 times higher than in white muscle, where Mb was 0.499±0.104 mg g^{-1} in normoxic fish and 0.361±0.075 mg g^{-1} in deeply hypoxic fish. When connected values of muscle nitrite and muscle Mb concentration from all individual fish are plotted against each other, it is evident that there is no linear correlation between muscle nitrite and Mb concentration, either when combining all groups or when analyzing them independently (Fig. 5). The main difference is that nitrite levels in deeply hypoxic red muscle are raised above the values in the other groups (Fig. 5).

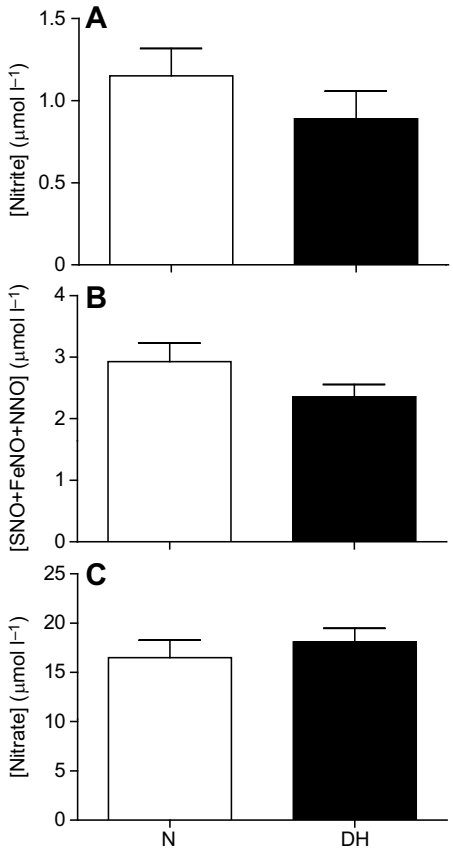

Fig. 1. Plasma nitric oxide (NO) metabolites in normoxic and deeply hypoxic crucian carp. Concentrations of nitrite (A), *S*-nitroso (SNO)+iron-nitrosyl (FeNO)+*N*-nitroso (NNO) compounds (B) and nitrate (C) in plasma of crucian carp after 1 day exposure to normoxia (N; P_{O_2}>140 mmHg) or deep hypoxia (DH; 1<P_{O_2}<3 mmHg). Values are means±s.e.m. (N=15 for each group).

In white skeletal muscle homogenates from both normoxic and deeply hypoxic fish, there was no significant nitrite production during 5 h hypoxic incubation in the absence of exogenous nitrate (Fig. 6A,E and C,G). However, when 300 µmol l^{-1} nitrate was added, the muscle tissue from deeply hypoxic fish showed a significant nitrite production (Fig. 6D,H). In muscle from normoxic fish, the change in nitrite concentration was not significant (P=0.07) (Fig. 6B,F). In the liver, we obtained similar results from N_2 incubations in Eppendorf tubes and in rotating tonometers with continuous flow of N_2 (Fig. 7). The nitrite production from nitrate in liver was significant after 5 h incubation in the presence of added nitrate (Fig. 7), and the nitrate reductase activity was higher than that in muscle. For comparison, we also tested nitrite production in a single mouse liver and found a 3 times higher production than in crucian carp (data not shown). The nitrite production in liver homogenates from crucian carp was significantly inhibited by allopurinol (Fig. 8). We did not observe nitrate reduction in the heart, and the nitrite concentration actually decreased by about 2 µmol l^{-1} during 5 h of incubation (Fig. 9).

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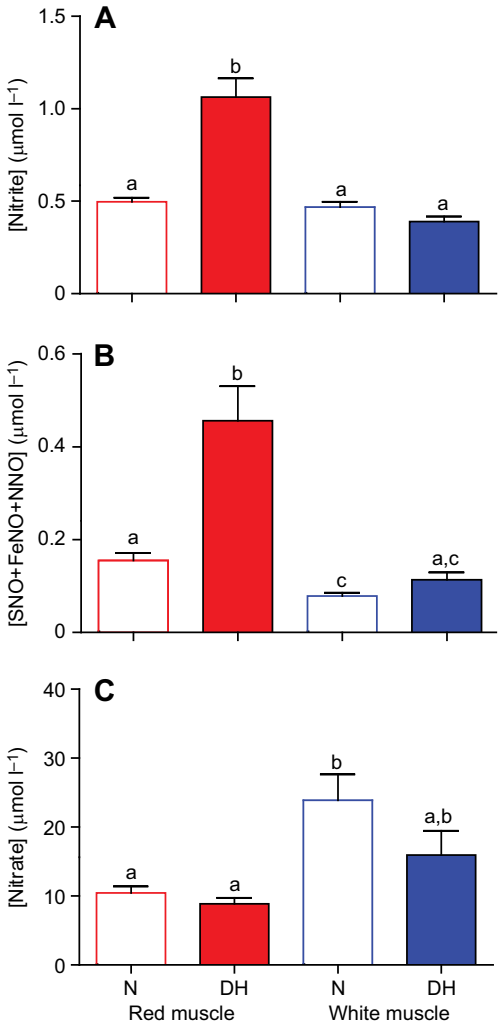

Fig. 2. Red and white muscle NO metabolites in normoxic and deeply hypoxic crucian carp. Concentrations of nitrite (A), SNO+FeNO+NNO (B) and nitrate (C) in red muscle and white muscle of crucian carp exposed to normoxic (N; P_{O_2}>140 mmHg) and deeply hypoxic (DH; 1<P_{O_2}<3 mmHg) water for 1 day. Values are means±s.e.m. (*N*=15 for each group). Differences between groups were tested using a one-way ANOVA, followed by a Bonferroni *post hoc* test, and different letters signify statistical differences between groups.

Ethanol was not detected in white muscle and plasma from normoxic fish, whereas ethanol increased to 10.5±1.1 mmol l^{-1} (mean±s.e.m., *N*=8) in muscle and 3.9±0.3 mmol l^{-1} (mean±s.e.m., *N*=8) in plasma of crucian carp exposed to deep hypoxia for 1 day.

DISCUSSION

A major finding of the current study is that tissue nitrate reduction can take place in crucian carp during deep hypoxia, indicating that nitrate reductase activity, albeit low, may participate in supplying nitrite for cytoprotection in the deeply hypoxic crucian carp. Furthermore, we found that deep hypoxia induces increased concentrations of nitrite and nitros(yl)ation compounds in red muscle but not white muscle. The Mb concentration was considerably higher in red muscle than in white muscle and it was unaffected by hypoxia. As discussed below, we suggest that tissue nitrate reductase activity supplements other nitrite supply

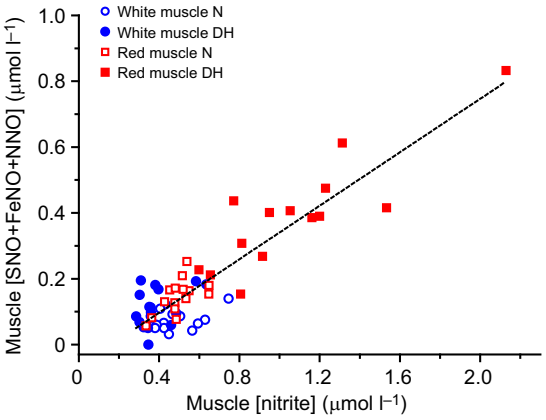

Fig. 3. Relationship between SNO+FeNO+NNO and nitrite concentration in muscle tissues from normoxic and deeply hypoxic crucian carp. Connected values of SNO+FeNO+NNO and nitrite concentration in red muscle (red symbols) and white muscle (blue symbols) of individual crucian carp exposed to normoxic (N; P_{O_2}>140 mmHg) and deeply hypoxic (DH; 1<P_{O_2}<3 mmHg) water. The dashed line represents the overall linear regression (y=0.406x−0.066, R^2=0.811, *N*=60).

routes in deeply hypoxic crucian carp, and that the Mb concentration is not directly responsible for increased tissue nitrite accumulation.

Mb and NO metabolites in red and white muscle

The present study is the first to examine NO metabolite and Mb levels in both red and white muscle in a hypoxia-tolerant lower vertebrate. We found considerably higher Mb concentrations in red muscle than in white muscle, and the level of nitrite increased significantly with exposure to deep hypoxia in red muscle, but not in white muscle. The latter observation corroborates the idea that tissues rich in Mb and mitochondria, such as red muscles and the heart, develop increased nitrite levels during severe O_2 deprivation (Jensen et al., 2014). Thus, hypoxia or anoxia has previously been found to increase nitrite concentration in pectoral muscle (having a high proportion of red muscle fibers) of slider turtles and in cardiomyocytes from slider turtles and crucian carp, whereas white

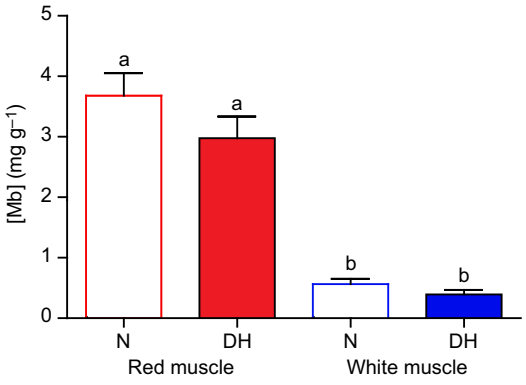

Fig. 4. Myoglobin (Mb) concentration in red and white muscle from normoxic and deeply hypoxic crucian carp. Mb concentration is given in mg per g wet mass for crucian carp exposed to normoxia (N; P_{O_2}>140 mmHg) and deep hypoxia (DH; 1<P_{O_2}<3 mmHg) for 1 day. Values are means±s.e.m. (*N*=15 for each group). Differences between groups were tested using a one-way ANOVA, followed by a Bonferroni *post hoc* test, and different letters signify statistical differences between groups.

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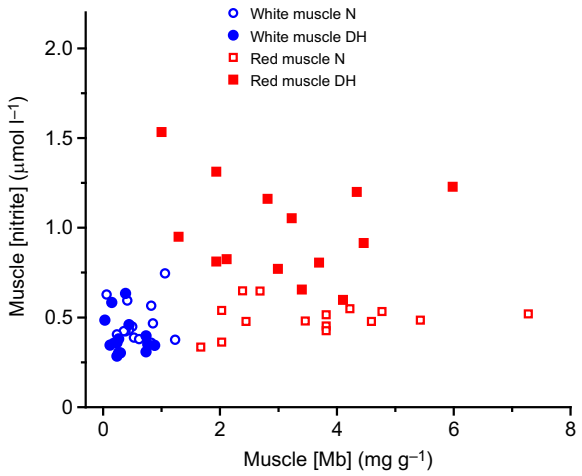

Fig. 5. Red and white muscle nitrite levels as a function of Mb concentration. Interrelated values of nitrite and Mb concentration in red muscle (red symbols) and white muscle (blue symbols) of individual crucian carp exposed to normoxia (N; P_{O_2}>140 mmHg) and deep hypoxia (DH; 1<P_{O_2}<3 mmHg). *N*=60 in total.

muscle nitrite concentration in crucian carp and goldfish was maintained or tended to drop (Hansen and Jensen, 2010; Sandvik et al., 2012; Jensen et al., 2014).

The hypoxia-induced increase in red muscle nitrite concentration was mirrored by an increase in nitros(yl)ation products (Fig. 2) and a strong linear correlation between muscle nitrite concentration and [SNO+FeNO+NNO] was found to apply to both normoxic and deeply hypoxic red and white myocytes (Fig. 3). This substantiates the nitrosative power of nitrite, where nitrite (via nitrosating species like N_2O_3) generates SNO compounds, and supports that NO originating from nitrite reduction nitrosylates heme groups to form FeNO compounds. The overall response of increased nitrite and nitros(yl)ation compounds in red muscle during deep hypoxia resembles the increased nitrite/NO activity found in cardiomyocytes from deeply hypoxic and anoxic crucian carp and slider turtles (Sandvik et al., 2012; Jensen et al., 2014; Hansen et al., 2016).

As outlined in the Introduction, we recently hypothesized that Mb may be involved in maintaining/increasing intracellular nitrite levels in hypoxia-tolerant species (Jensen et al., 2014), and the present study therefore tested for a correlation between muscle nitrite and Mb levels. The levels of Mb in red muscle and white muscle of normoxic crucian carp (Fig. 5) are similar to those reported in other fish species (Reynafarje, 1963; Giovane et al., 1980; Jaspers et al., 2014). Additionally, 1 day of deep hypoxia did not change the Mb protein level, which is in accordance with other studies on Mb expression during hypoxia, where 2–5 days of hypoxia in killifish, goldfish and carp did not affect Mb concentration in heart or red muscle (Cossins et al., 2009; Borowiec et al., 2015) or *Mb* mRNA levels (Roesner et al., 2008; Okogwu et al., 2014). If Mb concentration had a direct influence on muscle nitrite levels, one would expect a positive correlation between muscle nitrite and muscle Mb concentration. Such a correlation was clearly not present (Fig. 5). The data reflect an increased red muscle nitrite concentration that is independent of muscle Mb concentration (Fig. 5).

Other possible mechanisms for elevating intracellular nitrite levels should also be considered. As high mitochondrial content is also a shared property of red muscle and cardiomyocytes, mitochondria could be involved in elevating intracellular nitrite concentration during deep hypoxia. Sequestering of nitrite inside

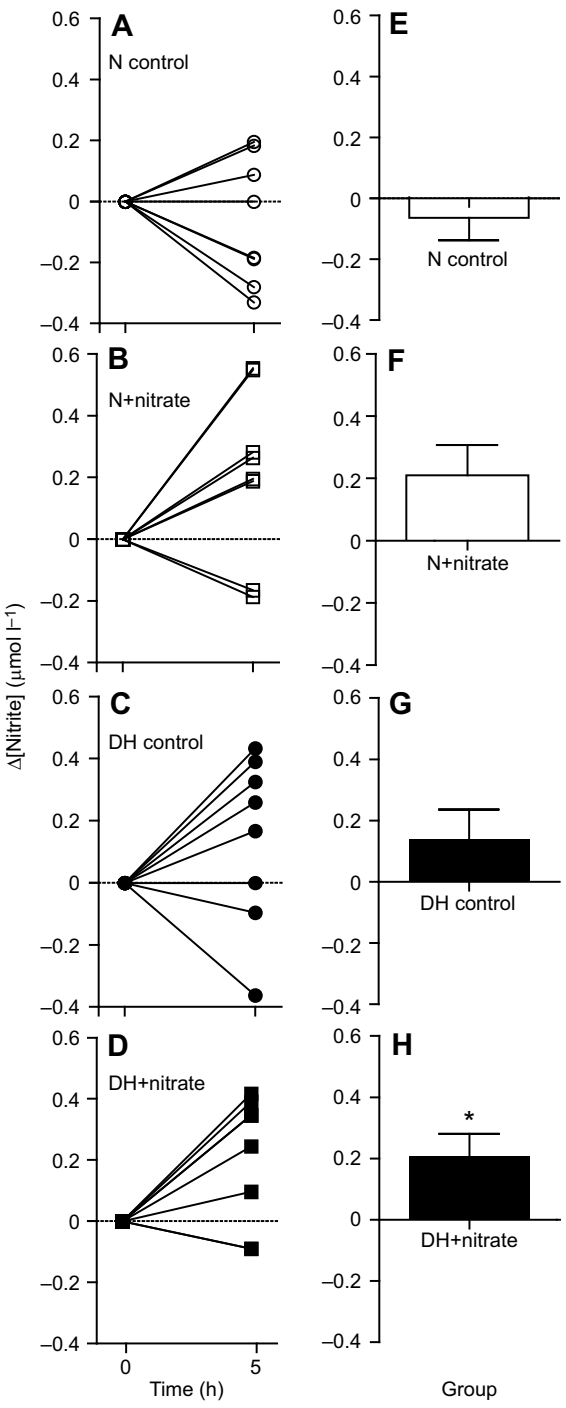

Fig. 6. Nitrate reductase activity in white muscle homogenates. Muscle homogenates are from normoxic (N) and deeply hypoxic (DH) crucian carp. (A–D) Change in nitrite concentration in individual samples during 5 h of N_2 incubation with (squares) or without (circles) 300 µmol l^{-1} exogenous nitrate added to the homogenates. (E–H) Group-specific changes as means±s.e.m. A one-sample *t*-test was performed to compare group means with zero; asterisks signify a statistical difference (*P*<0.05; *N*=8 for each group).

mitochondria would be much in line with the fact that the cytoprotective effects of nitrite are largely directed at the mitochondria (Halestrap, 2004; Walters et al., 2012; de Lima Portella et al., 2015). In the mitochondria, nitrite *S*-nitrosates

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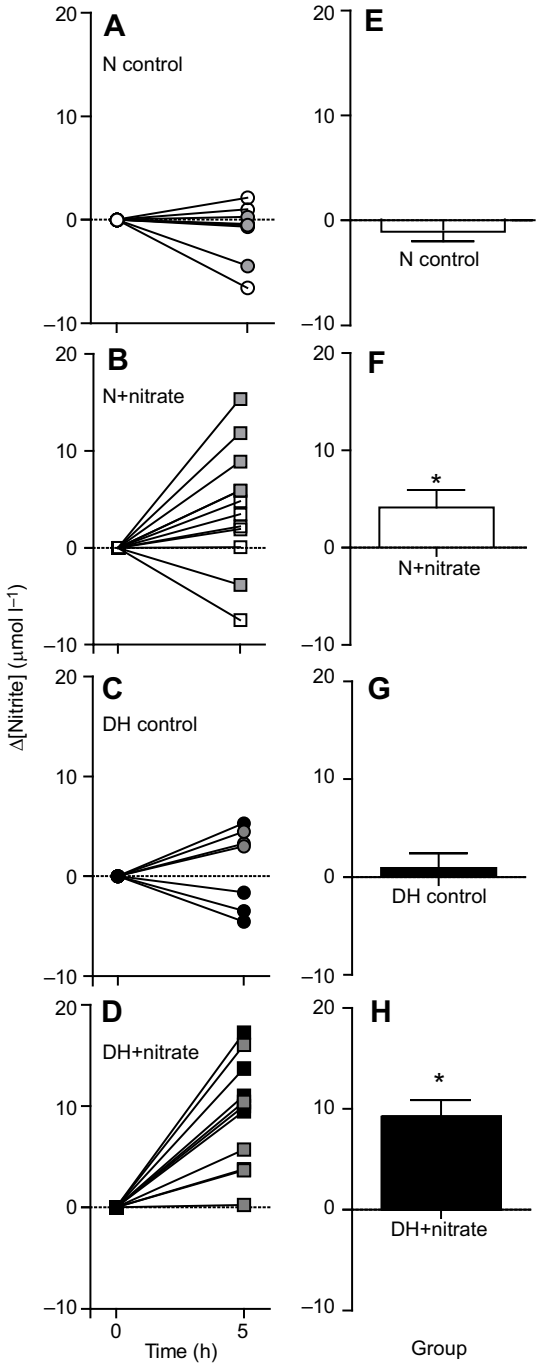

Fig. 7. Nitrate reductase activity in liver homogenates. Liver homogenates are from normoxic (N) and deeply hypoxic (DH) crucian carp. (A–D) Change in nitrite concentration in individual samples during 5 h of N_2 incubation with (squares) or without (circles) 300 μmol l^{-1} exogenous nitrate added to the homogenates. Black and white symbols are from incubations in Eppendorf tubes and gray symbols signify incubation in tonometers (see Materials and methods). (E–H) Group-specific changes as means±s.e.m. A one-sample *t*-test was performed to compare group means with zero; asterisks signify a statistical difference (P<0.05; N=7–11 for each group).

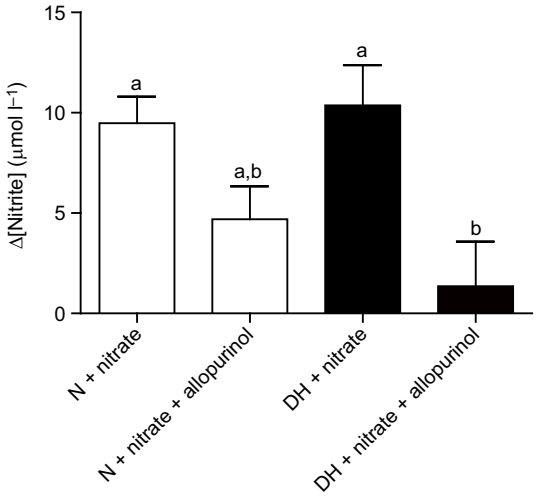

Fig. 8. Effect of allopurinol on nitrate reductase activity in liver homogenates. Liver homogenates are from normoxic (N) and deeply hypoxic (DH) crucian carp. All groups (means±s.e.m.) were incubated with 300 μmol l^{-1} nitrate for 5 h with or without 2 mmol l^{-1} allopurinol (N=5 in each group). Differences between groups were tested using a one-way ANOVA, followed by a Bonferroni *post hoc* test, and different letters signify statistical differences between groups.

complex I, attenuating ROS generation during early reperfusion (Dezfulian et al., 2009; Chouchani et al., 2013), and nitrosylates complex IV, which inhibits oxygen consumption rates (Hendgen-Cotta et al., 2008). However, a role of nitrite binding/storage by mitochondria is not known and future studies are needed to clarify the possible role of mitochondria in elevating cellular nitrite levels during hypoxia and anoxia.

Tissue nitrate reductase activity

The major sources of nitrate in the body are oxidation of NOS-generated NO (i.e. reaction with oxygenated heme proteins) and uptake from the diet, producing internal nitrate concentrations in the micromolar range well above nitrite concentrations (Lundberg et al., 2008). This larger pool of nitrate is a potential source of nitrite, provided nitrate can be reduced to nitrite in the tissues. Nitrate reduction was previously thought to be attributed solely to bacterial nitrate reductase enzymes and, thus, nitrate reduction in mammals was believed to be restricted to commensal bacteria in the oral cavity (Lundberg and Govoni, 2004). Recent studies have changed this belief and it is now known that mammals possess inherent tissue nitrate reductase activity that complements the more efficient nitrite reduction by oral bacteria (Li et al., 2003; Jansson et al., 2008; Huang et al., 2010; Piknova et al., 2015). The present study expands on this knowledge, by showing that among ectotherms, the crucian carp is also able to reduce nitrate to nitrite. The nitrate reduction activity was higher in liver (Fig. 7) than in muscle (Fig. 6) and it was absent in the heart (Fig. 9). In the heart, the nitrite concentration actually decreased, pointing at a dominating nitrite reduction under the assay conditions. We did not test for nitrate reductase activity in red muscle because the limited amount of red muscle tissue was totally consumed by the measurements of NO metabolites and Mb. However, given the low nitrate reduction activity in white muscle and its absence in the heart (resembling red muscle metabolically), the nitrate reduction activity may also be low in red muscle, which is supported by the absence of a decrease in red muscle nitrate concentration in deeply hypoxic fish (Fig. 2C). The higher nitrate reduction in the liver will supply nitrite to the anoxic liver, but it may additionally function to export nitrite to other tissues, including the heart, via the circulation, along with the function of the liver to

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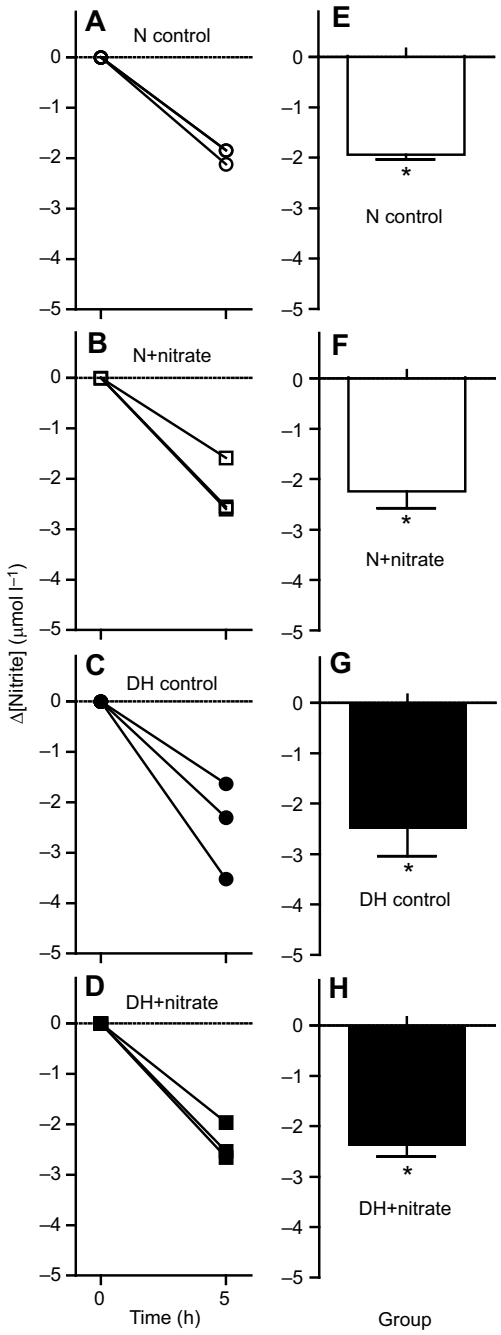

Fig. 9. Test for nitrate reductase activity in heart tissue. Ventricle homogenates are from normoxic (N) and deeply hypoxic (DH) crucian carp. (A–D) Change in nitrite concentration in individual samples during 5 h of N_2 incubation with (squares) or without (circles) 300 μmol l^{-1} exogenous nitrate added to the homogenates. (E–H) Group-specific changes as means±s.e.m. A one-sample *t*-test was performed to compare group means with zero; asterisks signify statistical difference (P<0.05). Because of the small ventricle size, ventricles from two fish were pooled in each individual homogenate (N=3 for each group).

deliver glucose to sustain glycolytic metabolic activity during deep hypoxia.

The difference in nitrate reductase activity between tissues corresponds to findings in mice and rats, where liver nitrate reductase activity is high compared with that in heart and muscle (Jansson et al., 2008; Piknova et al., 2015). Interestingly, in the current study, the nitrate reductase activity appeared higher in tissues from crucian carp exposed to deep hypoxia for 1 day than in tissues obtained from normoxic fish (Figs 6D,H and 7D,H), which suggests that the enzyme(s) responsible for nitrate reduction in crucian carp may be upregulated with transition to deep hypoxia. One main enzyme responsible for nitrate reduction is XOR, as reflected by a significant (but incomplete) inhibition of nitrate reductase activity using the XOR inhibitor allopurinol (Fig. 8). This is similar to mammals (Jansson et al., 2008; Piknova et al., 2015), where a role for XOR is further substantiated by the finding of high nitrate reductase activity in the liver and gastrointestinal tract (Jansson et al., 2008), which have the highest XOR levels (Harrison, 2004). In mammals, XOR expression is upregulated by hypoxia (Kelley et al., 2006), and also in germ-free mice, where nitrate reduction by commensal bacteria is absent (Huang et al., 2010), and XOR is known to also be expressed in teleost fish (Basha and Rani, 2003; Garofalo et al., 2015). Thus, XOR is a likely candidate for nitrate reductase activity in crucian carp, and given that the expression pattern is similar to that in mammals, this would explain the difference between nitrate reductase activity in crucian carp liver and muscle. An upregulation of XOR (and/or another nitrate reductase enzyme) in crucian carp would explain the increase in tissue nitrate reductase activity in tissues from deeply hypoxic fish compared with normoxic fish. It is also possible that other nitrate reductase enzymes, expressed solely during hypoxia, contribute to nitrate reduction in crucian carp. This awaits future study.

As a test of the nitrate reduction assay, we analyzed a mouse liver homogenate concurrently with crucian carp liver (data not shown), and found mouse liver nitrate reductase activity to be around 3 times higher than crucian carp liver nitrate reductase activity. Thus, there indeed appears to be a lower nitrate reduction activity in crucian carp tissue compared with mammalian tissue. An explanation could be that crucian carp utilize other sources of nitrite mobilization. Alternative nitrite sources in crucian carp, compared with mammals, are nitrite uptake from the ambient water (Hansen et al., 2016) and higher basal plasma nitrite levels (Fago and Jensen, 2015). Indeed, crucian carp take up ambient nitrite across the gills and direct it to tissues such as the heart during deep hypoxia (Hansen et al., 2016). By having access to an ambient pool of nitrite, crucian carp may be less dependent on internal nitrate reduction as a nitrite source. The ability to keep up internal nitrite levels is important for securing NO availability (e.g. nitrite reduction to NO) during deep hypoxia and anoxia, where NOS enzymes cannot produce NO because of the absence of O_2. A number of cellular proteins can reduce nitrite to NO, including deoxygenated Mb (Lundberg et al., 2008; Fago and Jensen, 2015). In the deeply hypoxic fish in the present study, the tissue oxygen tension will be practically zero and Mb will be deoxygenated and serve as an effective nitrite reductase.

Nitrate reductase activity in crucian carp muscle was low and insignificant in normoxic fish but significant in fish exposed to deep hypoxia. In rats, muscle nitrate reductase activity tended to increase after 24 h incubation with nitrate, and even though only low levels of nitrite were produced, it was suggested that muscle nitrate reductase activity could play a role in whole-body nitrite generation because of the large mass of skeletal muscle (Piknova et al., 2015). Likewise, the low levels of nitrite produced from nitrate in crucian carp muscle may sum to make a difference, because white muscle constitutes around 50% of the fish mass.

Concluding remarks

In summary, the main finding of the present study is the documentation of a low nitrate reductase activity in crucian carp tissues that supplements other nitrite supply routes and probably contributes to cytoprotection in deep hypoxia. Additionally, we found that red musculature – like cardiac musculature but in contrast to white musculature – increases nitrite levels and thus NO availability during deep hypoxia. This is not directly explained by high Mb levels and future studies will determine whether this increase in intracellular nitrite may relate to the high mitochondria content that characterizes cardiomyocytes and red muscle fibers.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

M.N.H., F.B.J. and J.O.L. conceived and designed the experiments; M.N.H., F.B.J., M.F., A.F. and N.M.G.C. performed the experiments; M.N.H., F.B.J., M.F., A.F. and N.M.G.C. analyzed the data; M.N.H. and F.B.J. wrote the paper; M.N.H., F.B.J., J.O.L., M.F., A.F. and N.M.G.C. revised the manuscript.

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Nesfatin-1 as a new positive inotrope in the goldfish (*Carassius auratus*) heart

R. Mazza [1], A. Gattuso [1], M. Filice, P. Cantafio, M.C. Cerra, T. Angelone, S. Imbrogno *

Dept. of Biology, Ecology and Earth Sciences (B.E.ST), University of Calabria, Arcavacata di Rende (CS), Italy

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ABSTRACT

The hypothalamic neuropeptide Nesfatin-1 is present in both mammals and teleosts in which it elicits anorexigenic effects. In mammals, Nesfatin-1 acts on the heart by inducing negative inotropism and lusitropism, and cardioprotection against ischemic damages. We evaluated whether in teleosts, Nesfatin-1 also influences cardiac performance. In the goldfish (*Carassius auratus*), mature, fully processed Nesfatin-1 was detected in brain, gills, intestine and skeletal muscle, but not in the cardiac ventricle. However, on the isolated and perfused working goldfish heart, exogenous Nesfatin-1 induced a positive inotropic effect, revealed by a dose-dependent increase of stroke volume (SV) and stroke work (SW). Positive inotropism was abolished by inhibition of adenylate cyclase (AC; MDL123330A) and cAMP-dependent kinase (PKA; KT5720), suggesting a cAMP/PKA-mediated pathway. This was confirmed by the increased cAMP concentrations revealed by ELISA on Nesfatin-1-treated hearts. Perfusion with Diltiazem, Thapsigargin and PD98059 showed the involvement of L-type calcium channels, SERCA2a pumps and ERK1/2, respectively. The role of ERK1/2 and phospholamban in Nesfatin-1-induced cardiostimulation was supported by Western blotting analysis.

In conclusion, this is the first report showing that in teleosts, Nesfatin-1 potentiates mechanical cardiac performance, strongly supporting the evolutionary importance of the peptide in the control of the cardiac function of vertebrates.

1. Introduction

Nesfatin-1 is an 82-amino acid peptide encoded in the precursor protein nucleobindin-2 (NUCB2), which is known to function as an anorexigenic peptide (Oh-I et al., 2006). It was originally characterized in rats where Nesfatin-1 immunoreactivity was detected in the hypothalamic nuclei involved in feeding behaviors, food intake, body weight, and glucose homeostasis (Elmquist et al., 2005), and also peripherally in the endocrine cells of the stomach, pituitary (Stengel et al., 2009), and pancreas (Stengel et al., 2009; Gonzalez et al., 2009). It was detected in rat cerebrospinal fluid (Oh-I et al., 2006) and in the peripheral circulation of both humans and rodents (Li et al., 2010; Su et al., 2010; Stengel et al., 2009).

As in the case of other peptides that regulate and/or are regulated by feeding and metabolism, Nesfatin-1 is known to influence cardiovascular function, *via* a central control of sympathetic and vagal influence on arterial blood pressure and heart rate (Mimee et al., 2012; Yosten and Samson, 2009). In parallel with this central action, the peptide elicits a direct influence on the cardiac performance. As shown on the rat heart, which expresses both Nesfatin-1 and the precursor NUCB2, the exogenous peptide depresses contractility and relaxation, and elicits cardioprotection against ischemia/reperfusion injuries (Angelone et al., 2013). Lastly, it affects cardiomyocyte energy metabolism, by increasing glucose uptake, like insulin (Feijóo-Bandín et al., 2013).

An appetite regulatory effect of Nesfatin-1 has been recently demonstrated also in fish. In the goldfish (*Carassius auratus*), a well characterized and widely used organism for studying aspects of molecular evolution and comparative genomics (Luo et al., 2006), cell biology (Lee et al., 1997), immunology (Hanington et al., 2006), neurobiology (Preuss et al., 2006; Huesa et al., 2005), and

Abbreviations: AC, adenylate cyclase; β-AR, beta adrenergic receptor; BSA, bovine serum albumin; CO, cardiac output; ERK1/2, extracellular signal-regulated kinases1/2; GAPDH, Glyceraldehyde-3-Phosphate Dehydrogenase; HCNP, hippocampal cholinergic neurostimulating peptide; HR, heart rate; ISO, Isoproterenol; NLT, nucleus lateralis tuberis; NUCB2, nucleobindin-2; pERK1/2, phosphorylated ERK1/2; PLN, phospholamban; pPLN, phosphorylated PLN; PKA, cAMP dependent kinase; SV, stroke volume; SW, stroke work.

* Corresponding author at: Dept of Biology, Ecology and Earth Sciences, University of Calabria, 87036 Arcavacata di Rende (CS), Italy.
E-mail address: sandra.imbrogno@unical.it (S. Imbrogno).

[1] These authors equally contributed to the work.

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cardiac physiology (Imbrogno et al., 2014; Garofalo et al., 2012), Gonzalez et al. (2010) recently revealed Nesfatin-1-like-immunoreactive cells in the hypothalamus, within the nucleus lateralis tuberis (NLT) and the anterior intestine, J-loop. They observed that brain NUCB2 mRNA expression, as well as Nesfatin-1 circulating levels decrease upon fasting and increase after meal intake (Gonzalez et al., 2010), clearly indicating an anorexigenic role also in fish. Similar to mammals, in goldfish Nesfatin-1 appears to be linked to other appetite regulatory peptides, since in brain and gut it co-localizes with ghrelin, and alters ghrelin, cholecystokinin and orexin mRNA expression (Kerbel and Unniappan, 2012).

In the present study, we aimed to analyze whether the cardiovascular properties of Nesfatin-1, already demonstrated in mammals, can be extended also to teleosts.

For this purpose, we used the goldfish (*C. auratus* L.) heart, whose structure and function were previously characterized by our morphological and physiological investigations (Garofalo et al., 2012). We demonstrated that, when perfused under basal conditions, the goldfish heart exhibits a volume pump mechanical behavior, showing a basal mechanical performance which remains stable for more than 2 h (Garofalo et al., 2012). Taking advantage of these characteristics, we designed the present study to investigate the effects elicited by exogenous mammalian Nesfatin-1 (about 60% homology with the goldfish Nesfatin-1 amino acid sequence; Gonzalez et al., 2010) on the basal cardiac performance of the goldfish. In parallel, we observed that the peptide is expressed in various tissues such as brain, gills, intestine and skeletal muscle. Our experiments showed that, on the isolated and perfused working goldfish heart Nesfatin-1 induces a positive inotropic effect mediated by a cAMP/PKA pathway, which involves L-type calcium channels, SERCA2a pumps and ERK1/2. Moreover, exposure of the perfused heart to Nesfatin-1 is accompanied by an increased ERK1/2 and phospholamban (PLN) phosphorylation.

2. Materials and methods

2.1. Animals

Specimens of goldfish *C. auratus* (mass = 54.0 ± 1.8 g; mean ± SEM), provided by a fish farm (COF SAS, Bologna, Italy), were kept at room temperature (18–20 °C) for 7–10 days. They were anesthetized with MS222 (tricaine methanesulfonate; 0.2 g/L) (Sigma–Aldrich, Italy), whereupon the heart was dissected out. Animal care and procedures were in accordance with the U.S. National Institutes of Health's Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1996), with the Italian law (DL 116, January 27, 1992), and with Directive 2010/63/EU.

2.2. Isolated and perfused in vitro working heart preparations

The hearts, removed without the parietal pericardium and cannulated, were connected to a perfusion apparatus as described by Imbrogno et al. (2001). The hearts received Ringer's solution from an input reservoir and pumped against an afterload pressure given by the height of an output reservoir. The Ringer's solution contained the following in mmol/L: NaCl 124.9, KCl 2.49, $MgSO_4$ 0.94, NaH_2PO_4 1, Glucose 5, $NaHCO_3$ 15, and $CaCl_2$ 1.2. The saline was equilibrated with a mixture of 99.5% O_2 and 0.5% CO_2. pH was adjusted to 7.7–7.9 (Imbrogno et al., 2001). Experiments were carried out at room temperature (18–20 °C). Pressures were measured with two MP-20D pressure transducers (Micron Instruments, Simi Valley, CA, USA) connected to a PowerLab data acquisition system and analyzed 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. Stroke volume (SV = CO/HR) was used as a measure of ventricular performance. Ventricular stroke work [SW; mJ/g; (afterload-preload) × SV/ventricle mass] served as an index of systolic functionality.

2.3. Experimental protocols

2.3.1. Basal conditions

Isolated and perfused hearts were allowed to maintain a spontaneous rhythm for up to 15–20 min. In all experiments, the control conditions were a mean output pressure of about 1.5 kPa, with a CO set to 10–12 ml/min/kg body mass by appropriately adjusting the filling pressure (Imbrogno et al., 2014; Garofalo et al., 2012).

The heart generated its own rhythm. Cardiac variables were measured simultaneously during experiments. To distinguish between inotropic and chronotropic effects, cardiac preparations were electrically paced. To this purpose, two electrodes were located in the perfusion chamber, near the heart, and connected with a 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, means ± SEM].

Hearts that did not stabilize within 20 min of perfusion (about 20% of the preparations) were discarded.

2.3.2. Drug application

After the 15–20 min control period, the hearts were perfused for 20 min with Ringer's solution enriched with Nesfatin-1 at increasing concentrations (from 0.0001 to 10 nmol/L) to construct cumulative concentration–response curves. The cardiac performance variables were measured after 10 min of perfusion with each concentration of the drug. Preliminary experiments showed that repeated exposure of each heart to a single concentration (0.01 nmol/L) of Nesfatin-1 did not cause desensitization (data not shown). Thus, concentration–response curves were generated by perfusing cardiac preparations with Ringer solution supplemented with increasing concentrations (from 0.0001 to 10 nmol/L) of the peptide.

To verify the pathways involved in the mechanism of action of Nesfatin-1, the hearts were stabilized for 15–20 min with Ringer solution and then perfused with Nesfatin-1 (0.01 nmol/L) for 10 min; after, they were washed with Ringer to return to control conditions and perfused with an inhibitor alone for 20 min; then, they were perfused with Ringer containing Nesfatin-1 (0.01 nmol/L) plus the specific inhibitor for additional 20 min. The involvement of adenylate cyclase (AC), cAMP-dependent kinase (PKA), L-type calcium channels, SERCA2a pumps and ERK1/2 was evaluated by perfusing the hearts with MDL123330A (100 nmol/L), or KT5720 (100 nmol/L), or Diltiazem (10 nmol/L), or Thapsigargin (100 nmol/L), or PD98059 (100 nmol/L), respectively.

Each experiment was completed within 2 h, i.e. before the hypodynamic state (Garofalo et al., 2012).

2.3.3. Western blotting and densitometric analysis

To evaluate Nesfatin-1 expression in different tissues, Western blotting analysis was performed on heart, brain, gills, intestine and skeletal muscle extracts. The different tissues were homogenized in ice-cold RIPA buffer (Sigma–Aldrich, Milan, Italy) containing a mixture of protease inhibitors (1 mmol/L aprotinin, 20 mmol/L phenylmethylsulfonyl fluoride, and 200 mmol/L sodium orthovanadate). Homogenates were then centrifuged at 200 g for

10 min at 4 °C to remove tissue debris. Bradford reagent was used to determine protein concentration according to the manufacturer (Sigma–Aldrich). Amounts of 50 μg protein of each tissue were electrophoresed through a reducing SDS/10% or SDS/15% (w/v) polyacrylamide gel and electroblotted on to a nitrocellulose membrane. The membrane was blocked and incubated overnight at 4 °C with rabbit polyclonal antibody against Nesfatin-1 (Phoenix Pharmaceuticals, Germany) diluted 1:500 in TBS-T containing 5% bovine serum albumin (BSA). The same protocol was applied to evaluate the phosphorylation levels of ERK1/2 (E-4) and phospholamban (PLN) (Ser16) on homogenates of hearts perfused with Nesfatin-1 (0.01 nmol/L) for 30 min. In this case, blots were blocked and incubated overnight at 4 °C with the rabbit polyclonal antibody against phosphorylated ERK1/2 (pERK1/2, SantaCruz Biotechnology, Germany) and phosphorylated phospholamban (pPLN, SantaCruz Biotechnology, Germany). Protein loading was verified using Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) for Nesfatin-1 detection, and total ERK1/2 and total PLN for pERK1/2 and pPLN, respectively.

The peroxidase linked secondary antibodies (anti-mouse) (GE Healthcare Europe GmbH, Italy) were diluted 1:5000 in TBS-T containing 5% non-fat dry milk. Immunodetection was performed by using an enhanced chemiluminescence kit (ECL PLUS, GE Healthcare Europe GmbH, Italy).

Autoradiographs were scanned to obtain arbitrary densitometric units. The experiments were performed in triplicate and the results expressed as mean ± SEM of absolute values.

2.4. ELISA assay

2.4.1. Measurements of Nesfatin-1 plasma levels

For determination of plasma level of Nesfatin-1, blood samples were collected through a needle inserted into the ventral aorta. The blood samples were treated with 6% trichloroacetic acid at 0 °C and centrifuged at 1000*g* for 10 min. Supernatants were extracted three times with 3 mL diethyl ether saturated with water, and the aqueous phases were collected and stored at −80 °C. In each multiwall plate, 100 μl of blood were loaded. Nesfatin-1 concentrations were measured by using commercial enzyme immunoassay kits (Nesfatin-1: ELISA Kit Protocol; Phoenix Pharmaceuticals, Germany).

2.4.2. cAMP and cGMP measurements

For cAMP and cGMP determination, frozen tissues extracts (200–300 mg) from control hearts and from hearts perfused with Nesfatin-1 (0.01 nmol/L) for 30 min were treated with 6% trichloroacetic acid at 0 °C and centrifuged at 1000*g* for 10 min. Supernatants were extracted three times with 3 mL diethyl ether saturated with water, and the aqueous phases were collected and stored at −80 °C [cAMP and cGMP Biotrack Enzyme Immunoassay (EIA) System; GE Healthcare Europe GmbH, Italy].

2.5. Drugs and chemicals

Nesfatin-1 was purchased from Phoenix Europe GmbH (Germany). MDL123330A and PD98059 were purchased from Sigma–Aldrich (Milan, Italy). KT5720, Diltiazem and Thapsigargin were purchased from Calbiochem (VWR International, Milan, Italy).

KT5720 and MDL123330A were prepared in DMSO; PD98059 was dissolved in ethanol; all the other drugs were dissolved in double-distilled water. Dilutions were made in Ringer's solution immediately before use.

Preliminary experiments showed that the presence of equivalent amounts of DMSO or ethanol in Ringer solution in the absence of any drug did not modify the basal cardiac performance.

2.6. Statistics

For data expressed as mean ± SEM of percentage changes obtained from individual experiments, statistical analysis was determined by using one-way ANOVA followed by Dunnett's post-test. Differences were considered statistically significant at $p < 0.05$.

For phospho blots and cAMP/cGMP measurements, expressed as means ± SEM of absolute values from individual experiments, statistical analysis was assessed by unpaired *t*-test. Statistical significance was concluded at $p < 0.05$. GraphPad Prism software, version 4.02 (GraphPad Software Inc., San Diego, CA, USA) was used for all the statistical analysis.

The concentration–response curve of the stimulation of SV induced by Nesfatin-1, fitted using GraphPad Prism 4.02 (GraphPad Software Inc., San Diego, CA, USA), provided the concentration (in −log M) of Nesfatin-1 that induced 50% of the maximum effect (EC_{50}).

3. Results

3.1. Tissues and plasma Nesfatin-1 identification

The expression of Nesfatin-1 in heart, brain, gills, intestine and skeletal muscle of *C. auratus* was evaluated by exposing extracts from these tissues to Nesfatin-1 antibody. Western blotting showed the presence of the peptide in brain [used as positive control (Oh-I et al., 2006)], gills, intestine, and skeletal muscle (Fig. 1), revealed by a band ~9.5 KDa, corresponding to the apparent molecular weight of Nesfatin-1. In contrast, Nesfatin-1-like immunoreactivity was not detected in ventricular cardiac extracts (Fig. 1).

ELISA test revealed a circulating Nesfatin-1 concentration of 4.8 ± 0.41 nmol/L.

3.2. Cardiac basal conditions

After 15–20 min of equilibration, baseline variables for the resting heart preparations were: SV = 0.18 ± 0.011 ml/kg; SW = 0.24 ± 0.006 mJ/g; HR = 55.6 ± 3.4 beat/min; preload = 0.16 ± 0.09 kPa; afterload = 1.49 ± 0.016 kPa and CO = 11.95 ± 0.32 ml/min/kg.

3.3. Nesfatin-1 effects on the basal cardiac performance

With the aim to evaluate whether exogenous Nesfatin-1 directly affects basal cardiac performance, we exposed isolated and perfused goldfish hearts to increasing concentrations (from 0.0001 to 10 nmol/L) of Nesfatin-1. The peptide induced a positive inotropic effect, revealed by a dose-dependent increase of SV and SW, significant starting from the concentration of 0.001 nmol/L (Fig. 2). The EC_{50} value (in log M) of Nesfatin-1 was −12.46 ± 0.33 ($r^2 = 0.65$) (Fig. 2).

3.4. Mechanism of action of Nesfatin-1

The transduction pathways responsible for the cardiac effects induced by Nesfatin-1 were studied with the application of specific inhibitors whose concentration was selected on the basis of the results of preliminary dose–response curves as the highest dose that did not significantly affect cardiac performance.

The involvement of adenylate cyclase (AC) was analyzed by perfusing goldfish cardiac preparations with Nesfatin-1 (0.01 nmol/L) in the presence of MDL123330A (100 nmol/L), a selective AC inhibitor. MDL123330A abolished the positive inotropic effect of

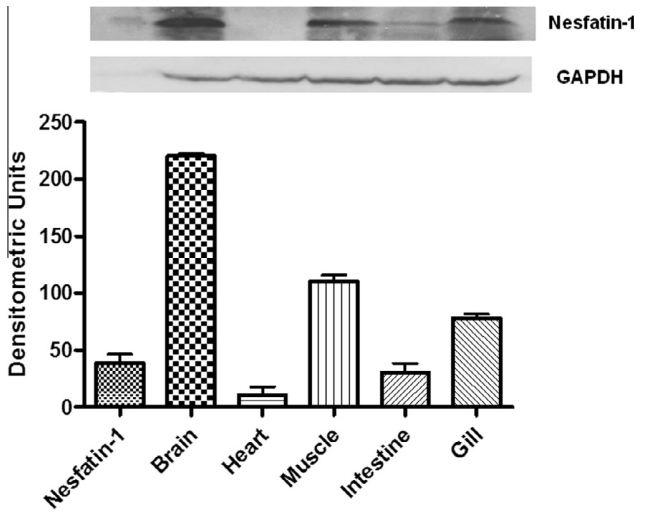

Fig. 1. Immunoblots of brain, heart, skeletal muscle, intestine and gills homogenates of goldfish showing expression of Nesfatin-1 and GPDH as housekeeping protein. First lane: Nesfatin-1. Values are mean ± SEM of four experiments for each group.

Nesfatin-1 (Fig. 3). A similar effect was obtained when cardiac preparations were perfused with KT5720 (100 nmol/L), a selective PKA inhibitor, in the presence of Nesfatin-1 (0.01 nmol/L) (Fig. 3).

To study the role of Ca^{2+}, the effects of Nesfatin-1 were tested before and after treatment with either the L-type calcium channels antagonist (Diltiazem, 10 nmol/L) or the SERCA2a pumps inhibitor (Thapsigargin, 100 nmol/L). Both inhibitors abolished the positive inotropic effect induced by Nesfatin-1 (Fig. 3). Moreover, the role of ERK1/2 in the Nesfatin-1-induced effects has been revealed by the ERK1/2 antagonist PD98059 (100 nmol/L). All inhibitors used *per se* did not modify the basal cardiac performance (Fig. 3).

3.5. cAMP and cGMP measurements

To test the involvement of cGMP and cAMP in the mechanism of action of Nesfatin-1, intracellular cAMP and cGMP concentrations were evaluated on goldfish ventricular extracts. Results showed that the treatment with Nesfatin-1 (0.01 nmol/L) significantly increased intracardiac cAMP levels, without changing cGMP levels (Fig. 4).

3.6. ERK1/2 and PLN phosphorylation

The involvement of the protein kinase ERK1/2 and PLN in the Nesfatin-1-dependent mechanism of action was evaluated by Western blotting. Results showed that exposure of the heart to Nesfatin-1 (0.01 nmol/L) significantly increased the phosphorylation of both ERK1/2 and PLN (Ser16) (Fig. 5).

4. Discussion

The present study is the first to analyze that, like in mammals, also in teleosts Nesfatin-1 is able to influence the basal mechanical performance of the heart. We found that Nesfatin-1 is present in various tissues such as brain, gills, intestine and skeletal muscle of the goldfish *C. auratus*. Although the goldfish heart does not express Nesfatin-1, the direct exposure of the isolated and perfused working heart to the exogenous peptide induced a dose-dependent positive inotropism which involves cAMP, PKA, L-type calcium channels and SERCA2a pumps, as well as ERK1/2 and PLN.

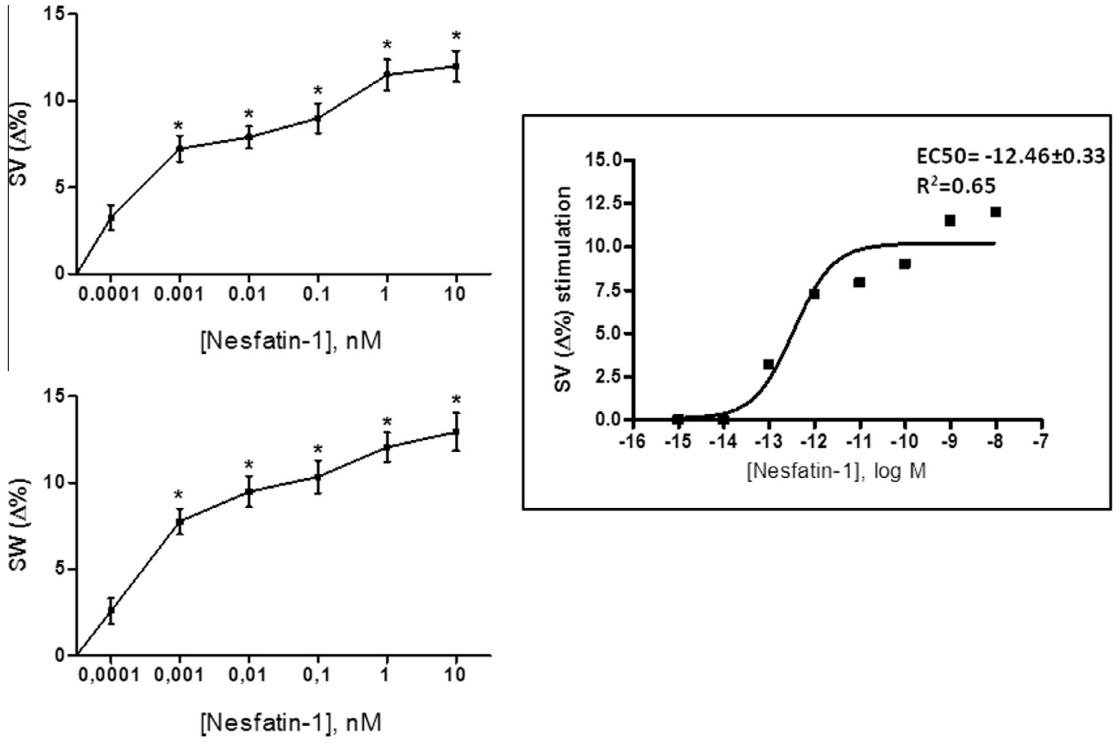

Fig. 2. Cumulative concentration–response curves of Nesfatin-1 on stroke volume (SV) and stroke work (SW) in isolated and perfused paced goldfish (*C. auratus*) heart. Percentage changes were evaluated as means ± SEM of four experiments. Significance of difference from control values (one-way ANOVA followed by Dunnett's post test): $^{*}p < 0.05$. The EC_{50} value (in log M) of Nesfatin-1 was −12.46 ± 0.33 ($r^2 = 0.65$).

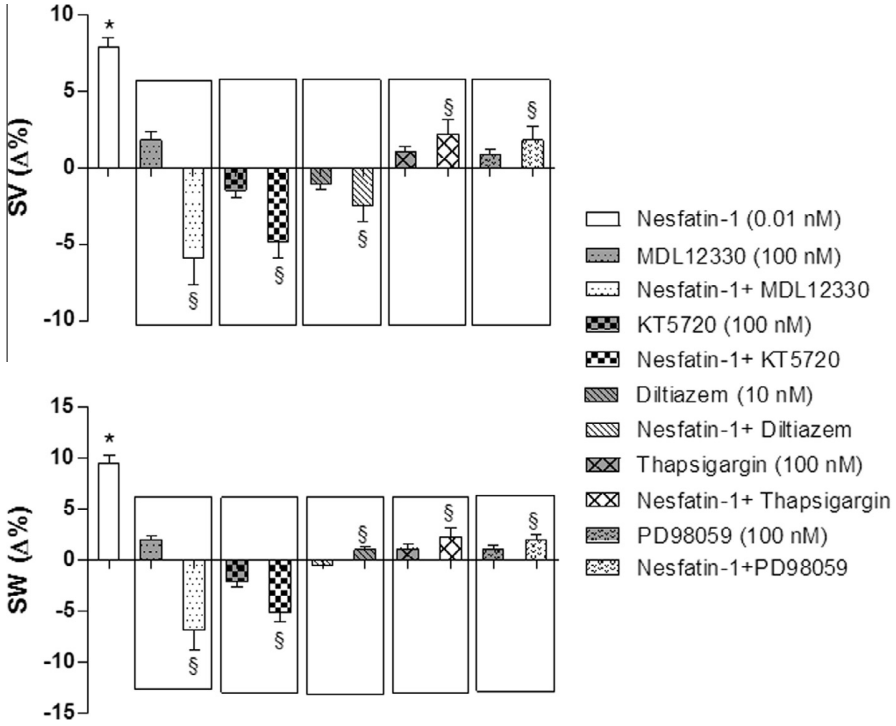

Fig. 3. Effects of Nesfatin-1 (0.01 nmol/L) before and after treatment with either MDL123330 (100 nmol/L), or KT5720 (100 nmol/L), or Diltiazem (10 nmol/L), or Thapsigargin (100 nmol/L), or PD98059 (100 nmol/L) on SV and SW in isolated and perfused paced goldfish hearts. The effects of all inhibitors used are also showed. Percentage changes were evaluated as mean ± SEM. Differences are indicated as: $^{*}p < 0.05$ (Nesfatin-1 *vs* control; $n = 4$) and $^{§}p < 0.05$ (Nesfatin-1 *vs* Nesfatin-1 + inhibitors; $n = 4$ for each group); one-way ANOVA followed by Dunnett's post hoc test.

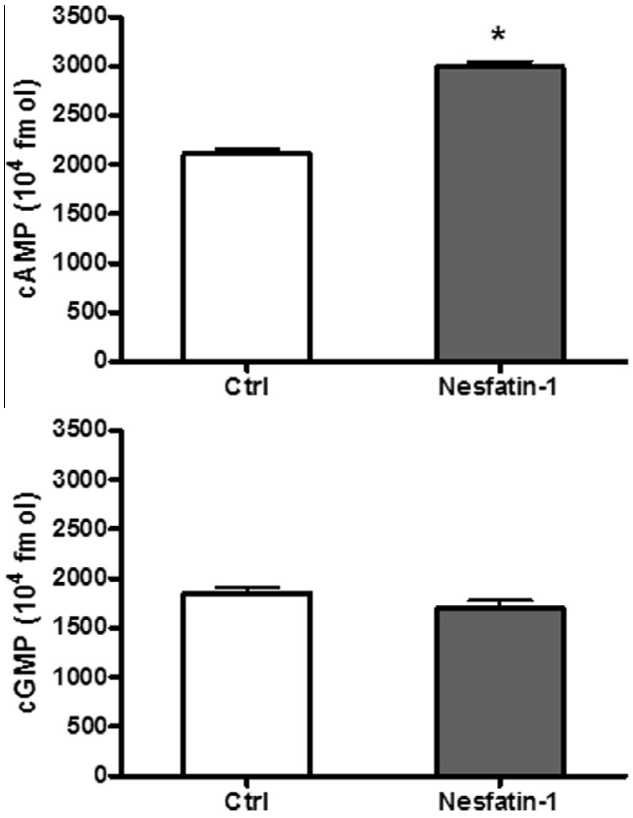

Fig. 4. cAMP and cGMP levels in control hearts and in hearts treated with Nesfatin-1 (0.01 nmol/L). Significance of difference from control values (unpaired *t*-test): $^{*}p < 0.05$ ($n = 3$).

4.1. Nesfatin-1 expression

A large number of investigations, mainly performed in rodents, established the broad distribution of NUCB2/Nesfatin-1 immunoreactivity in mammalian brain regions (see for example Goebel-Stengel et al., 2011; Foo et al., 2008), as well as in peripheral tissues, including pancreas (Gonzalez et al., 2009), gastric mucosa (Stengel et al., 2009), adipocytes (Ramanjaneya et al., 2010), gonads (Garcia-Galiano et al., 2012), blood (Tsuchiya et al., 2010), and heart (Angelone et al., 2013; Feijóo-Bandín et al., 2013). In 2010, Gonzalez et al. reported the expression of the NUCB2 mRNA in several tissues of a non-mammalian vertebrate, the teleost *C. auratus*. The highest levels were found in the liver and in several areas of the brain; lower levels were detected in adipose tissue, ovary, eye, kidney, and midgut, while the lowest levels were found in heart, muscle and gills (Gonzalez et al., 2010). Consistent with this distribution of the precursor, Western blotting analyses performed in the present investigation revealed that brain, gills, intestine and skeletal muscle of the goldfish express Nesfatin-1. However, contrarily to the evidence of a cardiac expression of NUCB2 in the goldfish heart (Gonzalez et al., 2010), the peptide was not detected in cardiac extracts (present study). This is of note since it suggests that, unlike mammals (Angelone et al., 2013; Feijóo-Bandín et al., 2013), the goldfish cardiac tissue does not represent a relevant site of Nesfatin-1 production. So far, the mature, fully processed peptide was identified in rat cerebrospinal fluid (Oh-I et al., 2006), and heart (Angelone et al., 2013), as well as in human plasma (Zhang et al., 2012). Since NUCB2 might be cleaved in a site which is different from that of its production, or might not be processed at all, in the absence of evidence on the presence of PC1/3 [i.e. the enzyme responsible for NUCB2 cleavage (Feijóo-Bandín et al., 2013)] in goldfish myocardiocytes, our results allow to hypothesize that in the *C. auratus* heart, NUCB2 is not

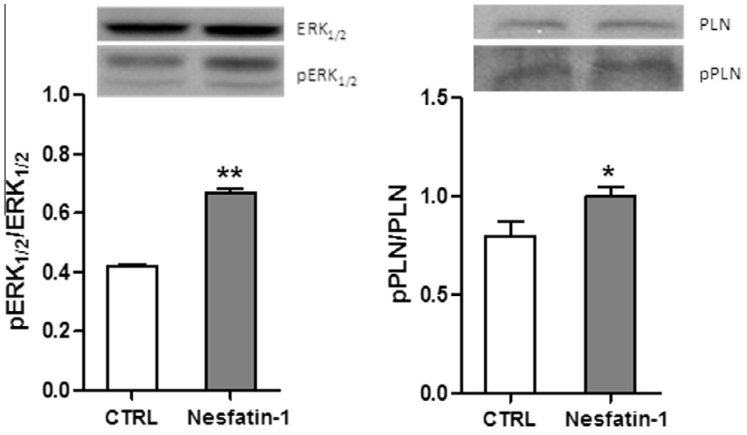

Fig. 5. Immunoblots of pERK1/2 and pPLN in control hearts and in hearts treated with Nesfatin-1 (0.01 nmol/L). Significance of difference from control values (unpaired *t*-test): $^{*}p < 0.05$; $^{**}p < 0.001$ ($n = 3$).

processed into Nesfatin-1. Alternatively, NUCB2 cleavage may generate an amount of Nesfatin-1 which is below the detectable range.

4.2. Nesfatin-1 as a cardioactive peptide

The results of the present study revealed that, although the goldfish heart does not generate Nesfatin-1, it is a target for the peptide. We observed that exposure of the isolated and perfused working cardiac preparations to exogenous Nesfatin-1 influenced the basal myocardial performance, as demonstrated by the dose-dependent increase of SV and SW. These effects are significant starting from the concentration of 0.001 nmol/L, which is lower than that detected by us in goldfish plasma (4.8 nmol/L), suggesting a very high cardiac sensitivity to the peptide. This is not surprising since, as shown in mammals, other neuropeptides affect myocardial performance at concentrations lower than those found in the blood [e.g. HCNP (Angelone et al., 2006)]. The findings of the present study are of interest since suggest that in teleosts, Nesfatin-1, in addition to appetite inhibition (Gonzalez et al., 2010), elicits a peripheral activity expressed by a direct potentiation of cardiac function. Notably, the goldfish is characterized by an extraordinary ability to face stressful conditions, as in the case of severe hypoxia (Bickler and Buck, 2007). Under these conditions, its heart responds by increasing the mechanical performance (Imbrogno et al., 2014). Whether Nesfatin-1 may contribute to the endogenous mechanisms of stress resistance in the goldfish remains a challenge for future studies.

The cardiostimulation observed on the isolated and perfused goldfish heart after exposure to Nesfatin-1 is in line with the central cardiovascular effects proposed for the peptide in mammals. In fact, hypertension and tachycardia were detected after microinjection of Nesfatin-1 within the cerebral ventricle, or in the medial nucleus of the solitary tract, a critically nervous site which receives projections from, and sends efferents to, multiple brain regions involved in the maintenance of cardiovascular homeostasis (Mimee et al., 2012; Yosten and Samson, 2009; Lawrence and Jarrott, 1996). These effects were attributed to a potentiation of the sympathetic outflow, since they were blocked by the adrenergic antagonist phentolamine (Yosten and Samson, 2009). In our experiments, the goldfish heart preparation was deprived of nervous control, and this reinforced the hypothesis of a direct peripheral action of the peptide. However, it cannot be excluded that, as in mammals, in *in vivo* teleosts the direct cardiac effects of Nesfatin-1 may be additive and/or alternative to a central action elicited on brain areas involved in the control of the cardiovascular function.

On the isolated and Langendorff perfused rat heart, the direct exposure to exogenous Nesfatin-1 induces a dose-dependent depression of contractility and relaxation (Angelone et al., 2013). The reason for this discrepancy is unknown. However, it is important to underline that the cardiac response to cardioactive agents is strongly influenced by species-specific differences. An example is the cardiac response to the non-specific beta adrenergic receptor (β-AR) agonist Isoproterenol that in mammals is known for its positive inotropic and chronotropic action (Brodde, 1991), while in fish induces also negative inotropic effects as a consequence of β3-AR stimulation (Imbrogno et al., 2006).

The analysis of the mechanism of action responsible for the positive inotropism elicited by Nesfatin-1 in the goldfish showed that exposure of the cardiac preparations to 0.01 nmol/L of the peptide is accompanied by a significant increase of cAMP, but not of cGMP, levels and this suggested a transduction mechanism mediated by AC. Consistent with this, AC inhibition with MDL123330A abolished the positive inotropism induced by the peptide. In turn, cAMP may activate PKA, whose involvement in the effects induced by Nesfatin-1 was suggested by the lack of cardiac stimulation observed after application of the specific inhibitor KT5720. As shown in mammals (Mattiazzi et al., 2005), important PKA targets are L-type calcium channels and SERCA2a pumps. Also in fish, both are involved in the rapid regulation of calcium transient during cardiac contraction and relaxation (see for references Garofalo et al., 2009; Imbrogno et al., 2004). In particular, L-type calcium channels are associated with a modulation of transmembrane calcium currents and contractile shortening amplitude (Vornannen, 1997). At the same time, SERCA2a pumps activation correlates to an increase of calcium reuptake into the SR, and thus of myocardial relaxation (Garofalo et al., 2009, and references therein). Myocardial calcium transients were not measured in the present study. However, it can be presumed that in the goldfish heart, Nesfatin-1 action occurs through modulation of Ca^{2+} cycles. Accordingly, inhibition of L-type calcium channels and SERCA2a pumps by Diltiazem and Thapsigargin, respectively, abolished Nesfatin-1-induced positive inotropism. The involvement of intracellular Ca^{2+} transients was also supported by PLN phosphorylation observed after Nesfatin-1 exposure. It is known that dephosphorylated PLN inhibits SR Ca^{2+} sequestration by SERCA2a (Kimura et al., 1997) but, when phosphorylated at Ser16 by PKA, the inhibition is relieved. This reduces cytosolic calcium overload, facilitates

myocardial relaxation and contributes to restore SR Ca^{2+} stores for subsequent contraction (Schmidt et al., 2001).

Of note, in the goldfish, Nesfatin-1 cardiostimulation was also accompanied by an increased phosphorylation of ERK1/2. This is in agreement with studies on murine and human cardiomyocytes in which Nesfatin-1 was found to recruit ERK1/2 to enhance glucose uptake (Feijóo-Bandín et al., 2013). The involvement of ERK1/2 in the mechanisms of action of Nesfatin-1 may be of relevance in relation to the well known stress resistance of the goldfish. In fact, as demonstrated in mammals, this kinase is involved in short- and long-term responses elicited by many cardioactive substances (Dube et al., 2006; Clerk and Sugden, 2004), and in cardioprotection against stress challenges, including ischemic damages (Darling et al., 2005).

5. Conclusions

In conclusion, by using physio-pharmacological and molecular approaches, we showed that Nesfatin-1 elicits a positive influence on the goldfish heart by increasing its contractility. This effect observed in *ex vivo* conditions, in the *in vivo* animal is presumably due to the extracardiac circulating peptide since in this teleost the heart is not a source for Nesfatin-1.

Notably, the effects of the peptide were associated to a pathway which involves intracellular mediators, known in mammals for their role in the modulation of basal cardiac performance, and in myocardial protection. Taking into account the increased heart performance that in the goldfish accompanies the exposure to stressful conditions, as hypoxia (Imbrogno et al., 2014), our results pave the way for the evaluation of Nesfatin-1 cardiostimulation as a neuroendocrine-dependent mechanism of the stress response in teleosts.

Funding

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Conflict of interest

None.

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Research paper

The Chromogranin A-derived sympathomimetic serpinin depresses myocardial performance in teleost and amphibian hearts

S. Imbrogno [a,*,1], R. Mazza [a,1], C. Pugliese [a], M. Filice [a], T. Angelone [a], Y.P. Loh [b], B. Tota [a], M.C. Cerra [a]

[a] Dept. of Biology, Ecology and Earth Sciences (B.E.S.T.), University of Calabria, Arcavacata di Rende (CS), Italy
[b] Section on Cellular Neurobiology, Eunice Kennedy Shriver National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD 20892, USA

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ABSTRACT

Chromogranin A (CgA) is an acidic protein co-stored with catecholamines, hormones and neuropeptides in the secretory granules of endocrine, neuronal and other cell types (including cardiomyocytes). Proteolytic cleavage in the C terminus of CgA generates a 2.9 kDa peptide named serpinin (Serp; Ala26Leu) that can be modified at its N terminus to form a pyroglutamate residue (pGlu-Serp). In the rat heart, both peptides increase contractility and relaxation through a β-adrenergic-like action mechanism. Accordingly, Serp and pGlu-Serp were proposed as novel myocardial sympatho-adrenergic modulators in mammals.

On a comparative basis, here we report the actions of Serp and pGlu-Serp on myocardial contractility in three poikilotherm vertebrate species: the eel (*Anguilla anguilla*), the goldfish (*Carassius auratus*) and the frog (*Rana esculenta*).

Using isolated working heart preparations, we show that pGlu-Serp reduces stroke volume in all species tested, while Serp reduces contractility in the frog heart, but is uneffective in eel and goldfish hearts. In the goldfish and frog hearts, pGlu-Serp activates the Nitric Oxide/cGMP pathway involving Endothelin-1 B receptors (frog) and β_3 adrenergic receptors (goldfish). pGlu-Serp-treated hearts from goldfish and frog show increased cGMP content. Moreover, the exposure of the frog heart to pGlu-Serp is accompanied by an increased expression of activated eNOS and Akt.

In conclusion, this first report showing that pGlu-Serp inhibits mechanical cardiac performance in teleost and amphibians supports an evolutionary role of the CgA system, and particularly its serpinin component, in the sympatho-adrenergic control of the vertebrate heart.

1. Introduction

Chromogranin A (CgA) is a major acidic protein of the secretory granules of the diffuse sympatho-chromaffin tissue (Winkler and Fischer-Colbrie, 1992) and is also present in endocrine, nervous and other cell types, including cardiomyocytes (Helle et al., 2007). It is co-stored in and co-released from the granules together with catecholamines (CAs), several hormones and neuropeptides (Winkler and Fischer-Colbrie, 1992). Identified almost five decades ago, CgA was extensively studied as to its expression, structure and function. The long evolutionary history of CgA is evidenced by its occurrence in many invertebrates such as coelenterates (Barkatullah et al., 1997), the nematode parasite *Ascaris suum* (Smart et al., 1992) and the protozoan *Paramecium tetraurelia* (Peterson et al., 1987). In vertebrates, CgA is ubiquitous and highly conserved. It is present in teleost fish (Deftos et al., 1987), amphibians (Reinecke et al., 1991), reptiles (Trandaburu et al., 1999), birds (Reinecke et al., 1991), humans, pigs and rats (Tota et al., 2007; Helle et al., 2007). CgA-positive cells have been detected in a number of mammalian species during early developmental stages in the adrenal medulla and the gastro-entero-pancreatic system together with various endocrine cells (Kent and Coupland, 1989; Mahata et al., 1993; Wang et al., 1994; Kameda et al., 1998). CgA-positive cells are also present in the brain and the dorsal root ganglia of the zebrafish (*Danio rerio*) (Xie et al., 2008), suggesting a

Abbreviations: β-ARs, β-adrenergic receptors; BSA, bovine serum albumin; CAs, catecholamines; CgA, Chromogranin A; CO, cardiac output; CST, Catestatin; ET-1, Endothelin-1; ET_AR, ET-1 type A receptors; ET_BR, ET-1 type B receptors; GC, guanylyl cyclase; HR, heart rate; NO, Nitric Oxide; NOS, Nitric Oxide Synthase; pAkt, phospho-Akt; peNOS, phospho-eNOS; pGlu-Serp, pyroglutamate serpinine; PI3K, PI3kinase; PKA, cAMP-dependent kinase; PKG, protein kinase G; PTx, pertussis toxin; Serp, serpinin; SV, stroke volume; SW, stroke work; VSs, Vasostatins.

* Corresponding author at: Lab of Organ Physiology, Dept. of Biology, Ecology and Earth Sciences, University of Calabria, 87036 Arcavacata di Rende (CS), Italy.
E-mail address: sandra.imbrogno@unical.it (S. Imbrogno).

[1] These authors equally contributed to the work.

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role during the early embryonic stages. Of cardiovascular relevance, studies performed during the last twenty-five years showed the presence of CgA also in the heart of a number of vertebrates. In the rat, it was identified in atrial myoendocrine granules (Steiner et al., 1990) and in the cardiac conduction system (Weiergraber et al., 2000). In humans, CgA was detected in the ventricular myocardium (Pieroni et al., 2007). Both in the human and rodent heart, it was found to be co-stored and co-secreted with CAs and natriuretic peptides (Steiner et al., 1990; Pieroni et al., 2007; Biswas et al., 2010). In the heart of non-mammalian vertebrates, CgA expression was detected in the secretory granules of frog atrial myocytes (Krylova, 2007).

CgA is a multifunctional protein which plays important biological roles. Within the (neuro)endocrine cells, in addition to the regulation of granule biogenesis (Courel et al., 2006), CgA functions as a prohormone for several biologically active peptides that control multiple homeostatic processes. These include the CgA-derived fragments Vasostatins (VSs) and Catestatin (CST) whose role as anti-adrenosympathetic stabilizers in cardiovascular homeostasis and anti-ischemic cardioprotection is under intensive investigations. VSs, the fragments corresponding to amino acids 1–76 (VS-1) and 1–113 (VS-2), and CST, a COOH-terminal fragment, in addition to their vascular effects, are now recognized as important cardiotropic modulators. In fact, in both mammalian and non mammalian vertebrates they exert anti-adrenergic cardiosuppressive actions for which they have been proposed as cardio-protective agents able to counteract the effects of excessive systemic and/or intra-cardiac excitatory stimuli [e.g. CAs and Endothelin-1 (ET-1)] (Tota et al., 2003; Corti et al., 2004; Imbrogno et al., 2004, 2010; Cerra et al., 2006; Angelone et al., 2008; Mazza et al., 2008, 2015a).

More recently, it has been shown that CgA cleavage at paired basic residues in its highly conserved C terminus, gives rise to a 2.9 kDa peptide named Serpinin (Serp; Ala26Leu) that can be modified at its N terminus to form a pyroglutamate residue (pGlu-Serp) (Koshimizu et al., 2011a,b). In neuroendocrine cells, Serp upregulates granule biogenesis *via* a cAMP-protein kinase A-Sp1 pathway (Koshimizu et al., 2011a), while pGlu-Serp inhibits cell death (Koshimizu et al., 2011b). Both Serp and pGlu-Serp have been proposed as novel CgA-derived cardiac modulators able to play a role in the myocardial response to sympathochromaffin stimulation. In fact, Tota and co-workers (2012) detected both fragments in the rat heart and used an *ex vivo* rat heart, Langendorff isolated and perfused preparation, to demonstrate that Serp and pGlu-Serp exert dose-dependent positive inotropic and lusitropic effects. Unlike the other CgA-derived cardioactive fragments (i.e. VS-1 and CST), which are well known Nitric Oxide (NO)-dependent negative anti-β adrenergic inotropes, both peptides activate a β1-adrenergic receptor (β1-AR)/adenylate cyclase (AC)/cAMP/cAMP-dependent kinase (PKA) pathway, acting as β-adrenergic-like agonists (Tota et al., 2012).

Very recently, employing the same rat heart preparation, Pasqua and co-workers (2015) have investigated the anti-ischemic cardioprotective potential of pGlu-Serp used in either pre-conditioning and post-conditioning experiments, showing in both conditions its striking implication in limiting ischemia-induced infarct size and myocardial failure.

In an evolutionary perspective, and with the aim to comparatively analyse the role of Serpinin peptides, we explored whether Serp and pGlu-Serp also affect myocardial contractility in three poikilotherm vertebrate species, namely the eel *A. anguilla*, the goldfish *C. auratus* and the frog *R. esculenta*.

We demonstrated that pGlu-Serp induces a reduction of contractility in all species tested. The pGlu-Serp-elicited effects appear mediated by a $G_{i/0}$/Akt/Nitric Oxide Synthase (NOS)/NO/cGMP/protein kinase G (PKG) signal transduction pathway and involve β3-ARs in goldfish and ET-1 B receptors (ET_BR) in frog. Taken together, these data suggest an early role of the CgA C-terminal Serpinin fragments in vertebrates, also highlighting the importance of species-specific action mechanisms.

2. Materials and methods

2.1. Animals

Specimens of eel (*A. anguilla* L.), goldfish (*C. auratus*) and frog (*R. esculenta*) of both sexes (body weight 97 ± 7 g, 55 ± 3 g and 21 ± 2 g, respectively) were used. Eels and frogs were provided by local hatcheries, while goldfish were provided by a fish farm (COF SAS, Bologna, Italy). All animals were kept at room temperature (18–20 °C) for 7–10 days. In accordance with the accepted standards of animal care, the experiments were organized to minimize stress and number of animals used. Animal care and procedures were in accordance with the U.S. National Institutes of Health's Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1996), with the Italian law (DL 26, March 4, 2014), and with Directive 2010/63/EU.

The experiments evaluating the effect of Serpinin peptides on the basal cardiac performance of the eel (*A. anguilla*) were performed in 2013. However, following the new Italian law about the care and use of Laboratory Animals, effective from March 2014, in which the European eel is considered an endangered species, this animal was excluded from further experiments designed to investigate the mechanism of action of the peptides.

2.2. Isolated and perfused in vitro working heart preparations

Eel and goldfish were anesthetized in benzocaine (0.2 g/L) for 15 min. After sacrifice, the heart was dissected out, removed by the pericardium, cannulated and connected with a perfusion apparatus (Imbrogno et al., 2001; Garofalo et al., 2012). For the eel heart, the perfusate composition (in mM) was: 115.17 NaCl, 2.03 KCl, 0.37 KH_2PO_4, 2.92 $MgSO_4$, 0.5 $(NH_4)_2SO_4$, 1.27 $CaCl_2$, 5.55 glucose, and 1.90 Na_2HPO_4 (Imbrogno et al., 2001). The goldfish heart was perfused with a Ringer's solution containing (in mM): NaCl 124.9, KCl 2.49, $MgSO_4$ 0.94, NaH_2PO_4 1, Glucose 5, $NaHCO_3$ 15, and $CaCl_2$ 1.2 (Garofalo et al., 2012). In both cases, saline was equilibrated with a mixture of 99.5% O_2 and 0.5% CO_2 and the pH was adjusted to 7.7–7.9 by adding $NaHCO_3$. Frogs were pithed and ventrally opened; the pericardium was removed and the heart, cannulated *in situ*, was connected to a perfusion apparatus (Gattuso et al., 1999). Frog Ringer solution contained (in mM): 115 NaCl, 2.5 KCl, 1.0 $CaCl_2$, 2.15 Na_2HPO_4, 0.85 NaH_2PO_4, and 5.6 glucose. Saline was equilibrated with air. pH was adjusted to 7.3 by adding Na_2HPO_4.

Experiments were carried out at room temperature (18–20 °C). Hemodynamic parameters were measured as previously reported (Gattuso et al., 1999; Imbrogno et al., 2001; Garofalo et al., 2012). 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. Stroke volume (SV = CO/HR) was used as a measure of ventricular performance. Ventricular stroke work [SW; mJ/g; (afterload-preload) × SV/ventricle mass] served as index of systolic functionality.

2.3. Experimental protocols

2.3.1. Basal conditions

Isolated and perfused hearts were allowed to maintain a spontaneous rhythm for up to 15–20 min. In all experiments, the control conditions were: mean output pressure 3.00 kPa with CO set

to 10–12 ml/min kg body mass (*A. Anguilla*: Imbrogno et al., 2001, 2010); mean output pressure 1.5 kPa with CO set to 10–12 ml/min/kg body mass (*C. auratus*: Garofalo et al., 2012; Imbrogno et al., 2014); mean output pressure 3.92 kPa with CO set to 110 ml/min/kg body mass (*R. esculenta*: Gattuso et al., 1999; Mazza et al., 2008).

Cardiac variables were measured simultaneously during experiments. To distinguish between inotropic and chronotropic effects, cardiac preparations were electrically paced. To this purpose, two electrodes were located in the perfusion chamber, near the heart, and connected with a LE 12006 stimulator (frequency identical to that of control, non-paced hearts). Hearts that did not stabilize within 15–20 min from the onset of perfusion were discarded.

2.3.2. Drug application

After the 15–20 min control period, hearts from eel, goldfish and frog were perfused with Ringer's solution enriched with either pGlu-Serp or Serp at increasing concentrations (11–33–69–110 nM) to generate cumulative concentration–response curves. The peptides used for the study were from mouse (NP_031719.1) and showed 59.3% identity and 74.1% similarity with zebrafish (NP-001006059.2) and 65.4% identity and 92.3% similarity with *Xenopus* (NP_001088193.1). The effects of the peptides remained stable until 15–20 min. Accordingly, cardiac performance variables were measured after 10 min of perfusion with each concentration of the drugs.

To elucidate the pathways involved in the mechanism of action of pGlu-Serp, hearts were stabilized for 15–20 min with Ringer solution before the perfusion with pGlu-Serp (33 nM) for 10 min; then they were washed with Ringer to return to control conditions and perfused with a specific inhibitor for 20 min. Subsequently, they were perfused with Ringer containing pGlu-Serp (33 nM) plus the inhibitor for additional 20 min.

The concentration-response curves of the SV variations induced by pGlu-Serp were fitted using GraphPad Prism 4.02. This provided the −log of the concentration (in M), which induced the 50% inhibition (IC50) of pGlu-Serp on SV.

In both frog and goldfish hearts, the involvement of NOS, guanylyl cyclase (GC), $G_{i/o}$-proteins, PKG, PI3kinase (PI3K), ET_AR, and ET_BR, β1/β2 and β3-AR was evaluated by perfusing the hearts with L-NMMA (10 μM), or ODQ (10 μM), or pertussis toxin (0.01 nM; PTx), or KT_{5823} (0.1 μM), or wortmannin (0.01 nM), or BQ_{123} (0.1 μM), or BQ_{788} (0.1 μM), or nadolol (0.1 μM) or SR_{59230} (10 nM), respectively. In the case of PTx experiments, hearts were pre-incubated for 60 min with the toxin, according to Imbrogno and co-workers (2003).

The concentration of the specific inhibitors was selected on the basis of preliminary dose-response curves as the highest dose that did not significantly affect cardiac performance.

Each experiment was completed within 2 h, i.e. before the hypodynamic state (eel: Imbrogno et al., 2001; goldfish: Garofalo et al., 2012; frog: Gattuso et al., 1999).

2.3.3. Western blotting and densitometric analysis

To evaluate the putative effects of pGlu-Serp on the cardiac expression of eNOS, phospho-eNOS (peNOS), Akt and phospho-Akt (pAkt), both untreated and pGlu-Serp-treated frog hearts (n = 5 for each group) were rapidly immersed in liquid nitrogen and stored at −80 °C. Subsequently, the hearts were homogenized in ice-cold RIPA buffer (Sigma Aldrich, Milan, Italy) containing a mixture of protease inhibitors (1 mM aprotinin, 20 mM phenylmethylsulfonyl fluoride and 200 mM sodium orthovanadate). Homogenates were centrifuged at 1500*g* for 10 min at 4 °C to remove debris. Protein concentration was determined by using Bradford reagent, according to the manufacturer (Sigma-Aldrich). For western blotting analysis of eNOS and Akt, proteins were separated on 8% SDS-PAGE gels, transferred to membrane, blocked with nonfat-dried milk, and incubated overnight at 4 °C with polyclonal rabbit anti-antibody [polyclonal rabbit anti-eNOS antibody (Sigma) or polyclonal rabbit anti peNOS antibody (Ser1177), or polyclonal rabbit anti-Akt antibody, or polyclonal rabbit anti pAkt1/2/3 antibody (Ser473) (Santa Cruz Biotechnology, Santa Cruz, CA)] diluted 1:500 in TBS-T containing 1% nonfat dried milk. Peroxidase linked anti-rabbit secondary antibody (Santa Cruz Biotechnology) was diluted 1:1000 in TBS-T containing 5% nonfat dried milk.

Protein loading was verified by using total eNOS, for peNOS, and total Akt for pAkt. Peroxidase-linked secondary anti-rabbit antibodies (Amersham) were diluted 1:2000 in TBS-T containing 5% non-fat dry milk. Immunodetection was performed by using an enhanced chemiluminescence kit (ECL PLUS, Amersham). Autoradiographs were obtained by exposure to X-ray films (Hyperfilm ECL, Amersham). Immunoblots were digitalized and the densitometric analysis of the bands was carried out using WCIF ImageJ based on 256 Gy values (0 = white; 256 = black). Quantification of the bands was obtained by measuring (8 times on each band) the mean optical density of a square area. Normalization between gels and blots was performed subtracting the background values from each measurement.

2.3.4. cAMP and cGMP measurements

For cAMP and cGMP determination, frozen tissue extracts (200–300 mg) from control hearts and from hearts perfused with pGlu-Serp (11–33–69–110 nM) were treated with 6% trichloroacetic acid at 0 °C and centrifuged at 1000*g* for 10 min. Supernatants were extracted three times with 3 mL diethyl ether saturated with water, and the aqueous phases were collected and stored at −80 °C [cAMP and cGMP Biotrack Enzyme Immunoassay (EIA) System; GE Healthcare Europe GmbH, Italy].

2.3.5. Drugs and chemicals

Serpinin peptides [wild-type (WT)-Ala26Leu, pGlu-Serp], were custom synthesized by Phoenix Pharmaceuticals, Inc. (Burlingame, CA, USA). NG-monomethyl-L-arginine acetate salt (L-NMMA), 1H-(1,2,4)oxadiazolo-(4,3-a)quinoxalin-1-one (ODQ), pertussis toxin (PTx), nadolol, 3-(2-ethylphenoxy)-1-[[(1*S*)-1,2,3,4-tetrahydro naphth-1-yl]amino]-(2*S*)-2-propanol oxalate salt (SR_{59230}), KT_{5823}, N-*cis*-2,6-dimethylpiperidinocarbonyl-L-gammamethylleucyl-D I-methoxycorbonyltryptophanyl-D norleucine, Na (BQ_{788}) and cyclo (L-Asp-Pro-L-Val-Leu-L-Trp) (BQ_{123}) were purchased from Sigma-Aldrich (Milan, Italy). Wortmannin was purchased from Calbiochem (VWR International, Milan, Italy). ODQ and BQ_{788} were prepared in DMSO; the other drugs were dissolved in double-distilled water. Dilutions were made in Ringer's solution immediately before use. Preliminary experiments showed that the presence of equivalent amounts of DMSO (0.01–0.1%) in Ringer solution in the absence of any drug did not modify the basal cardiac performance.

2.3.6. Statistics

For data expressed as means ± SEM of percentage changes obtained from individual experiments, statistical analysis was determined by using one-way ANOVA followed by Dunnett's post-test. Differences were considered statistically significant at $p < 0.05$.

For densitometric analyses, values were expressed as means ± SEM of absolute values from individual experiments; statistic was assessed by unpaired *t*-test. Significance was concluded at $p < 0.05$. GraphPad Prism software, version 4 (GraphPad Software, San Diego, CA) was used for all the statistical analysis.

3. Results

After stabilization, baseline cardiac hemodynamic parameters for eel, goldfish and frog are those reported in Table 1.

3.1. Serpinin peptides depressed contractility

To determine whether Serp or pGlu-Serp affects basal cardiac performance of eel, goldfish and frog, cardiac preparations were exposed to increasing concentrations (11–33–69–110 nM) of the peptides to generate concentration-response curves. In all species, pGlu-Serp caused a concentration-dependent negative inotropism, significant from 33 nM (Fig. 1). In contrast, Serp had no effect on the eel and goldfish hearts, but reduced SV from the concentration of 69 nM on the frog heart (Fig. 1). IC_{50} values of pGlu-Serp, evaluated in eel, goldfish and frog were: −7.4 ± 0.08 log M; R2 = 0.99 (eel), −7.9 ± 0.04 log M; R2 = 0.99 (goldfish), −7.78 ± 0.09 log M; R2 = 0.99 (frog).

To verify the specificity of Serpinin peptides effects, cardiac preparations from eel, goldfish and frog were exposed to the bovine serum albumin (BSA; 11–33–69–110 nM) used as inert peptide. In all preparations, treatments did not modify basal mechanical performance (data not shown).

3.2. Action mechanism of pGlu-Serp

3.2.1. Adrenergic and Endothelin-1 receptors

Whether pGlu-Serp act on the heart *via* classic receptor-ligand interactions remains unresolved. Using the Langendorff perfused rat heart, Tota and co-workers (2012) showed that pGlu-Serp, acting as allosteric β-ARs modulator, activates the classical cascade triggered by β-adrenergic agonists. To analyse the role of adrenergic receptors in the pGlu-Serp-induced cardiac effects, heart preparations from goldfish and frog were perfused with pGlu-Serp (33 nM) in the presence of either 0.1 μM of nadolol (β1/β2-ARs inhibitor), or 10 nM of SR_{59230} (β3-ARs antagonist). In both species, no significant differences were detected between untreated and nadolol-treated preparations, suggesting that the negative inotropism induced by pGlu-Serp was independent from β1/β2-ARs (Fig. 2). However, unlike the frog heart, in the goldfish heart, pretreatment with SR_{59230} blocked the effects induced by pGlu-Serp, thus suggesting the involvement of β3-ARs in the pGlu-Serp-induced cardio-suppression (Fig. 2).

While in fish no information is available, it is known that in the frog (*R. esculenta*) heart, ET_BR are involved in the cardioactive effect induced by the CgA fragment CST (Mazza et al., 2008). To investigate the involvement of ET-1 receptors in the inhibitory action of pGlu-Serp, cardiac preparations of both goldfish and frog were exposed to either BQ_{123} (0.1 μM), ET_AR antagonist, or BQ_{788} (0.1 μM), ET_BR antagonist. In the goldfish heart, neither treatment influenced the response to pGlu-Serp; contrarily, in the frog this response was abolished by BQ_{788} but not by BQ_{123}, suggesting the involvement of ET_BR (Fig. 2).

All the above treatments alone did not modify the basal cardiac performance (data not shown).

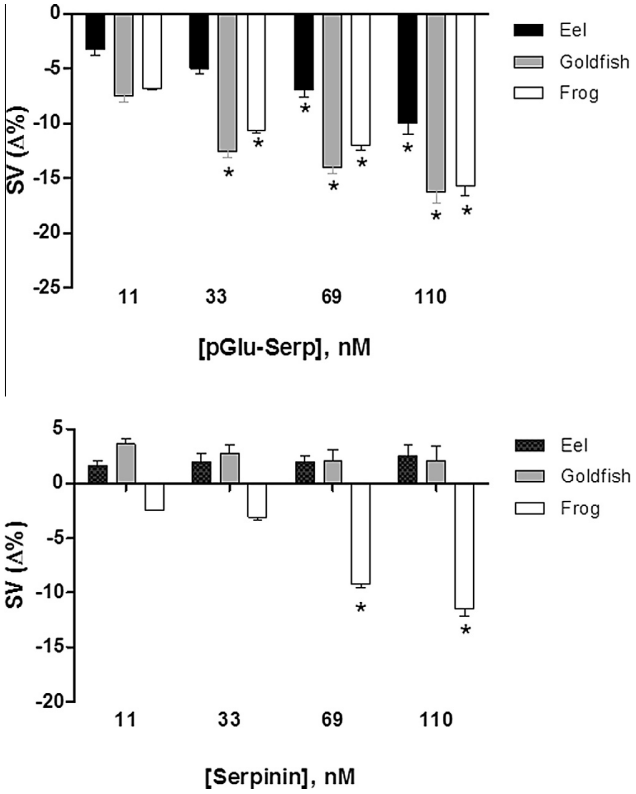

Fig. 1. Cumulative concentration–response curves of pGlu-Serp and Serp on stroke volume (SV) in isolated and perfused paced eel, goldfish and frog hearts. Percentage changes were evaluated as means ± SEM of five experiments for each group. Significance of difference from control values (one-way ANOVA followed by Dunnett's post test): $^{*}p < 0.05$.

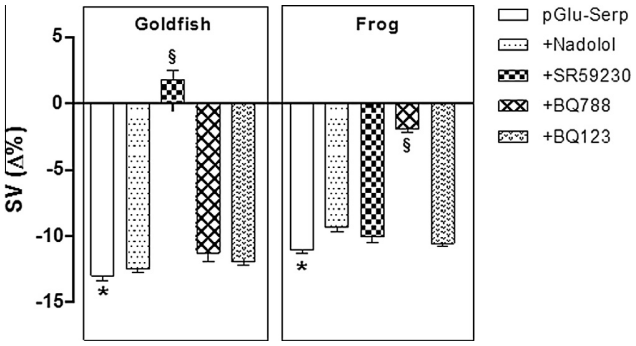

Fig. 2. Effects of pGlu-Serp (33 nM) before and after treatment with either nadolol (0.1 μM), or SR_{59230} (10 nM), or BQ_{788} (0.1 μM), or BQ_{123} (0.1 μM) on SV in isolated and perfused paced goldfish and frog hearts. Percentage changes were evaluated as means ± SEM. Differences are indicated as: $^{*}p < 0.05$ (pGlu-Serp *vs* control; n = 5–6 for each group) and $^{§}p < 0.05$ (pGlu-Serp + inhibitors *vs* pGlu-Serp; n = 5–6 for each group) (one-way ANOVA followed by Dunnett's post hoc test).

3.2.2. Gi/o proteins and NO-cGMP-PKG signal transduction pathway

G-proteins transduce many biological signals in intracellular responses. With the aim to study their putative involvement in the depressive contractile action of pGlu-Serp (33 nM), cardiac

Table 1
Performance variables under basal conditions of isolated eel, goldfish and frog hearts.

	SV (ml/kg)	SW (mJ/g)	HR (beats/min)	Preload (kPa)	Afterload (kPa)	CO (ml/min/kg)
Eel	0.21 ± 0.03	0.64 ± 0.12	61.5 ± 0.9	0.08 ± 0.006	2.85 ± 0.72	10.8 ± 1.2
Goldfish	0.19 ± 0.004	0.25 ± 0.015	56.27 ± 2.64	0.13 ± 0.05	1.48 ± 0.026	11.89 ± 0.25
Frog	1.75 ± 0.1	3.65 ± 0.24	58.15 ± 1	0.15 ± 0.006	3.9 ± 0.019	109.73 ± 2.53

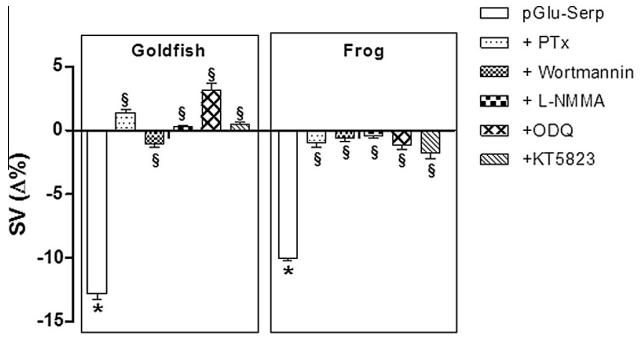

Fig. 3. Effects of pGlu-Serp (33 nM) before and after treatment with either PTx (0.01 nM), or wortmannin (0.01 nM), or L-NMMA (10 μM), or ODQ (10 μM), or KT_{5823} (0.1 μM) on SV in isolated and perfused paced goldfish and frog hearts. Percentage changes were evaluated as mean ± SEM. Differences are indicated as: $^{*}p < 0.05$ (pGlu-Serp *vs* control; n = 5–6 for each group) and $^{§}p < 0.05$ (pGlu-Serp + inhibitors *vs* pGlu-Serp; n = 5–6 for each group) (one-way ANOVA followed by Dunnett's post hoc test).

preparations from both goldfish and frog were preincubated with Ringer's solution containing PTx (0.01 nM) (Ai et al., 1998, and references therein).

In both species, PTx alone did not influence the mechanical performance (data not shown), whereas pre-treatment with the toxin abolished the pGlu-Serp-induced inotropism, suggesting a role for $G_{i/o}$ proteins (Fig. 3).

The role of NO as a major coordinator of many chemically activated cascades has been well established in mammals, as well as in non mammalian vertebrates (see for example for fish: Imbrogno et al., 2011; Imbrogno, 2013; for amphibians: Sys et al., 1997; Gattuso et al., 1999). This pathway is involved in the depressive contractile action elicited by the luminal exposure to VS-1 and CST (eel: Imbrogno et al., 2004, 2010; frog: Mazza et al., 2008). To analyse whether the cardiac response induced by pGlu-Serp involves the NO-cGMP-PKG pathway, heart preparations from goldfish and frog were pretreated with either L-NMMA (10 μM; NOS inhibitor) or ODQ (10 μM; guanylyl cyclase blocker) or KT_{5823} (0.1 μM; PKG antagonist). In both species, all treatments abolished the pGlu-Serp-induced basal inotropism, suggesting their reliance on a NOS/NO-cGMP-PKG signal transduction mechanism (Fig. 3).

Since it is known that the major mechanism of eNOS activation is its phosphorylation at serine-1177 residue (Ser1177) by serine–threonine kinase Akt (Dimmeler et al., 1999), we studied the heart response to pGlu-Serp also in the presence of the Akt inhibitor wortmannin, (0.1 nM). The inhibitor abolished the effects of the pGlu-Serp peptide (Fig. 3).

All the above mentioned inhibitors *per se* did not modify the basal cardiac performance (data not shown).

3.2.3. cAMP and cGMP measurements

The first identified target for NO is the soluble guanylate cyclase (sGC). NO binding to the heme group of sGC leads to increased conversion of GTP to cGMP. In turn, cGMP activates PKG which, through phosphorylation of troponin I, reduces the affinity of troponin C for calcium, thereby negatively regulating cardiac contractility (Hove-Madsen et al., 1996). Consistent with pGlu-Serp signaling through cGMP, we found increased cGMP content in pGlu-Serp-treated hearts from both goldfish and frog (Fig. 4). In both kind of preparations, exposure to pGlu-Serp did not influence cAMP intracellular levels (Fig. 4).

3.2.4. peNOS and pAkt expression

Western Blotting analysis of cardiac homogenates obtained from untreated and pGlu-Serp-treated frog hearts, exposed to

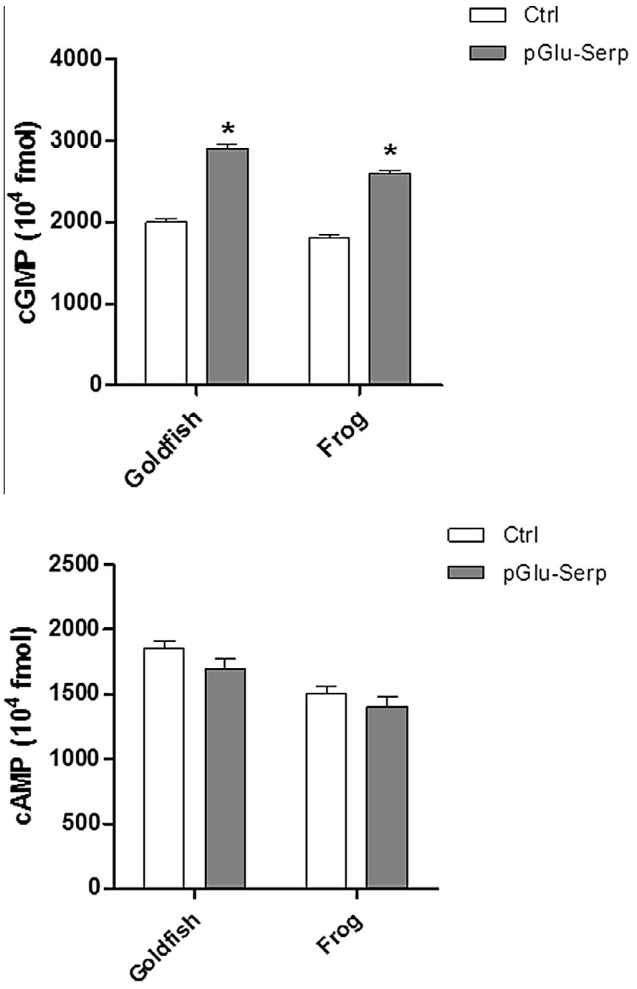

Fig. 4. cGMP and cAMP levels in control and pGlu-Serp (33 nM)-treated goldfish and frog hearts. Significance of difference from control values (unpaired *t*-test): $^{*}p < 0.05$ (n = 5).

polyclonal anti-eNOS, anti-peNOS, anti-Akt, and anti-pAkt antibodies, showed the expression of eNOS, peNOS, Akt and pAkt. This was revealed by the presence of immunoreactive bands of 135 kDa (eNOS), 140 kDa (peNOS), and 60 kDa (Akt and pAkt). peNOS and

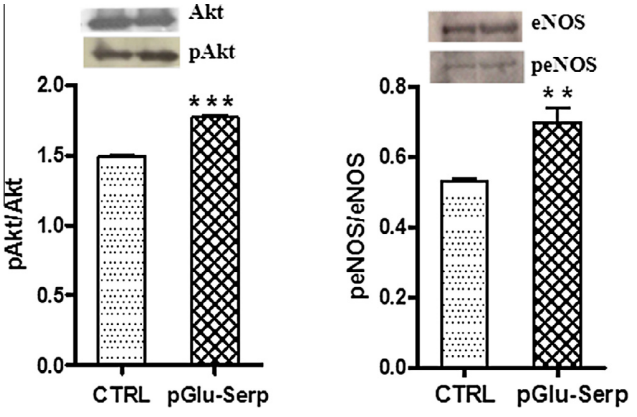

Fig. 5. Western blotting and densitometric analysis of phospho-protein kinase B (pAkt/Akt) and phospho-endothelial nitric oxide synthase (peNOS/eNOS) in control and pGlu-Serp-treated frog hearts (n = 5 for each group). Significance of difference from control values (unpaired *t*-test): $^{**}p < 0.001$; $^{***}p < 0.0001$.

pAkt expression was normalized with respect to anti-eNOS and anti-Akt expression, respectively. Densitometric quantification of the blots revealed that, with respect to the basal conditions (untreated hearts), the exposure to pGlu-Serp was accompanied by an increased expression of peNOS (29.59 ± 4.62%) and pAkt (18.56 ± 1.68%) (Fig. 5).

4. Discussion

The present study is the first to demonstrate the influence of the CgA C-terminal fragments Serp and pGlu-Serp on the cardiac performance of the teleosts *A. anguilla* and *C. auratus*, and the amphibian *R. esculenta*. The pyroglutaminated form of Serp induced a dose-dependent reduction of contractility in all species, whereas Serp was effective only on the frog heart. In *C. auratus* and *R. esculenta*, the pGlu-Serp-dependent depression of mechanical performance involved the NO-cGMP-PKG signal transduction pathway, and occurred *via* ET_BR in frog and β3-ARs in goldfish.

4.1. Serpinin effects on the basal cardiac performance

The exposure of perfused working heart preparations of eel, goldfish and frog to increasing concentrations of either Serp or pGlu-Serp showed that these CgA C-terminal fragments induced a significant cardio-suppressive influence. We also found that the inhibitory effect on contractility was more evident in the presence of pGlu-Serp than Serp. In fact, in all species tested, pGlu-Serp caused a concentration-dependent reduction of contractile performance, significant from the concentration of 33 nM, whereas Serp was effective only in the frog heart, in which it reduced SV starting from a higher concentration (69 nM). The magnitude of Serpinin effects (changes of approximately 10–15%) is in the same range of that reported for the CgA-derived fragments VS-1 and CST (see for example, Imbrogno et al., 2004, 2010 for eel; Mazza et al., 2007, 2008 for frog), the full length CgA (Pasqua et al., 2013), and other cardioactive agonists (e.g. nesfatin-1: Mazza et al., 2015a,b; Isoproterenol: Tota et al., 2004), whose biological significance in the modulation of cardiac performance is well established. In particular, we previously showed in the teleost *A. anguilla* (Imbrogno et al., 2004, 2010) and in the frog *R. esculenta* (Corti et al., 2004; Mazza et al., 2008), that the peptides VS-1 and CST exert dose-dependent negative contractile effects which started at 33 nM (∼15%), reaching a maximum (by ∼20%) at the highest concentration tested. On both species, the two peptides are also able to counteract the influence of physiological cardio-stimulating factors (e.g. catecholamines and Endothelin-1) (Imbrogno et al., 2004, 2010; Corti et al., 2004; Mazza et al., 2008). This is in agreement with the idea that CgA generates different hormone peptides which work as homeostatic stabilizers of a number of physiological processes, as illustrated for example by Koeslag and co-workers (1999). This would be of physiological importance under conditions of excessive adrenergic stimulations, such as under stress conditions in which CgA peptides counterbalance the effects of Isoproterenol and Endothelin 1 (see Tota et al., 2004; Mazza et al., 2008, for references). Interestingly, on the isolated and Langendorff perfused rat heart, the exposure to similar concentrations of pGlu-Serp induced a dose-dependent increase of contractility and relaxation (Tota et al., 2012). The reason for this divergence in the cardiac effects of Serpinin peptides in mammalian *vs* non-mammalian vertebrates is unknown. However, this is not surprising since the response to cardio-active agents is strongly influenced by species-specificity. For example, the non-specific β-AR agonist Isoproterenol is known to induce a positive inotropic and chronotropic action in mammals (Brodde, 1991), however in fish, in addition to the classic positive inotropism, it induces a negative inotropic effect, in which it acts *via* β3-AR stimulation (Imbrogno et al., 2006). Similarly, the anorexigenic peptide Nesfatin-1 induces a dose-dependent depression of contractility and relaxation on the rat heart (Angelone et al., 2013), but it increases the mechanical cardiac performance of the goldfish heart (Mazza et al., 2015b).

The circulating concentrations of Serpinin peptides in eel, goldfish and frog are unknown. In mammals (including humans), normal plasma levels of the precursor CgA are ∼5–10 nmol l^{-1} (O'Connor et al., 2002), thus below the concentrations required by CgA fragments to be effective on the heart. However, as suggested by rat data, the heart is a site for CgA processing into fragments which include Serpinin peptides (Tota et al., 2012; Pasqua et al., 2013). This local production was suggested to contribute to the cardiac effects induced by circulating fragments (Pasqua et al., 2013). Studies are needed to verify the hypothesis that like in mammals the heart of fish and amphibians could generate CgA fragments, including Serpinin peptides, which, in combination with the effects induced by the circulating peptides, may locally contribute to the fine-tuned modulation of the cardiac function (Tota et al., 2012; Pasqua et al., 2013). Moreover, the possibility that, as suggested in the rat heart (Pasqua et al., 2013), several CgA-derived peptides are released simultaneously and possibly act in concert must be taken into account.

This study did not analyse the effects of Serp peptides on spontaneously beating heart preparations. In mammals, although some CgA-derived peptides (e.g. CST and VSs) are reported to influence HR (Angelone et al., 2008; Cerra et al., 2006) and to counteract the chronotropism induced by ISO (Cerra et al., 2006), no effects of Serp peptides on HR have been highlighted (Tota et al., 2012).

4.2. Adrenergic and Endothelin-1 receptors involvement

So far, except for CST, which is known to interact with nicotinic receptors, functioning as a non-competitive antagonist (Mahata et al., 1997; Kraszewski et al., 2015), no conclusive evidence is available concerning the presence of specific receptors for CgA fragments, including Serpinin peptides. Therefore, whether Serpinin peptides act on the heart *via* classic receptor-ligand interactions still remains to be elucidated. Evidence in mammals suggests that pGlu-Serp influences cardiac basal performance by recruiting β1-AR (Tota et al., 2012). Our data obtained in the goldfish *C. auratus* and in the frog *R. esculenta* suggest that the pGlu-Serp-induced negative inotropism involved different receptor types in a specie-specific manner. In fact, in the goldfish the negative contractile action induced by pGlu-Serp involved β3-AR, since it was abolished by the β3-AR inhibitor SR_{59230}, but was unaffected by the ET_BR blocker BQ_{788}. Differently, in the frog, the peptide-induced effects involved ET_BR, being abolished by BQ_{788}, and unaffected by SR_{59230}. The reason for these differences is unknown. Both β3-AR and ET_BR are present in amphibian (Mazza et al., 2008, 2010) and in teleost (Imbrogno et al., 2006; Vierimaa et al., 2006) hearts. ET_B-R are responsible for ET-1-elicited cardio-depression in the frog (Mazza et al., 2008). However, unlike amphibians, the ET-1-dependent inotropic effect observed in salmon is mediated by ET_A-R (Vierimaa et al., 2006). Interestingly, in the frog, previous studies showed a link between ET_BR and CgA-derived peptides. In fact, this ET-1 receptor was found to mediate the negative cardiotropic actions of CST (Mazza et al., 2008) as well as the CST-dependent modulation of the Frank–Starling response (Mazza et al., 2012).

Functional β3-ARs were detected in the heart of teleosts (Nickerson et al., 2003; Imbrogno et al., 2006) and amphibians (Skeberdis et al., 2008; Mazza et al., 2010), in which they elicit a negative modulation of the contractile performance, also inhibiting the classic Isoproterenol-dependent positive inotropism (Imbrogno et al., 2006; Mazza et al., 2010). In the eel, β3-AR were found to be involved in the CST-evoked cardio-suppression (Imbrogno et al.,

2010). Accordingly, the two CgA fragments CST and pGlu-Serp share an action mechanism which requires β3-AR activation. Our data are of relevance since they disclose aspects of uniformity and diversity in the mechanism of action of CgA fragments among vertebrates, and emphasize the biological relevance of the third type of β-ARs in the fish heart modulation (Imbrogno et al., 2015).

4.3. NOS/NO pathway activation

The importance of NO as a crucial regulator of mammalian cardiac biology is well established. Growing evidence indicated that in non-mammalian vertebrates the intracardiac NOS-NO system also plays a major modulatory role. However, controversy still exists concerning several basic aspects. For example, while the presence of the eNOS gene and the cardiac expression of eNOS mRNA have been demonstrated in amphibians (Trajanovska and Donald, 2011), the eNOS gene appears absent in fish, as also documented by teleost genomes (Andreakis et al., 2011). On the other hand, physio-pharmacological studies with NOS inhibitors, as well as NADPH-diaphorase and immunolocalization with heterologous mammalian antibodies revealed the presence of "eNOS-like" activity in various piscine tissues (for references see Imbrogno et al., 2011; Andreakis et al., 2011), including the heart (Garofalo et al., 2009; Imbrogno et al., 2011). According to Andreakis et al. (2011), it is presumable that in fish, a neuronal NOS (nNOS) isoform including an endothelial-like consensus may cover some functional behaviors typical of the eNOS isoform identity. Studies mainly performed by our group documented, both in fish and amphibians, a widespread expression of a constitutive eNOS-like isoform, also highlighting the role of the eNOS/NO axis as a key autocrine/paracrine coordinator of complex physically and chemically activated intracellular signaling (see Imbrogno et al., 2011 for references). Notably, the present data indicated the involvement of NO also in the pGlu-Serp-induced cardiac effects in both goldfish and frog in which the depressive pumping performance induced by the peptide was affected by pretreatment with either PTx ($G_{i/o}$), or wortmannin (PI3K/Akt), or L-NMMA (NOS), or ODQ (sGC), or KT_{5823} (PKG). Consistent with this pGlu-Serp/NOS interaction, we found in the pGlu-Serp-treated hearts a parallel enhancement of cGMP intracellular concentration (goldfish and frog) and p-Akt and p-eNOS expression (frog). This reinforced our hypothesis that in both teleosts and amphibians, similarly to the other CgA-derived fragments so far analyzed (Imbrogno et al., 2004, 2010; Mazza et al., 2008), pGlu-Serp could induce, *via* a PI3K/Akt-dependent pathway, the release of NO which, by enhancing cGMP intracellular levels, exerts its cardio-suppressive inotropic influence.

5. Conclusions

Using physio-pharmacological and molecular approaches, this work showed, for the first time, that the newly discovered CgA-derived Serpinin peptides, in particular the pyroglutaminated form pGlu-Serp, elicit a negative influence on the contractile performance of the eel, goldfish and frog heart. These data are of relevance since they highlight the evolutionary importance of CgA and its C-terminal fragments in the modulation of the vertebrate cardiac performance. At the same time, the opposite cardiac effects induced by Serpinin peptides in mammals (e.g. β1-like positive inotropism: Tota et al., 2012) and in non-mammalian species (e.g. reduced pumping activity: present work) open new issues aimed to elucidate the role of Serpinin peptides in the vertebrate cardiac physiology. Are these opposite effects only the result of species-specific peculiarities? This should not be a surprise since it is known that the response to cardioactive agents is influenced by species-specific differences. Moreover, do the different cardiac effects induced by Serpinin peptides in vertebrates represent aspects of a still unknown evolutionary pattern? If this is the case, moving from non-mammalian species to mammals, the cardiac modulatory activity of the CgA fragments appears to differentiate

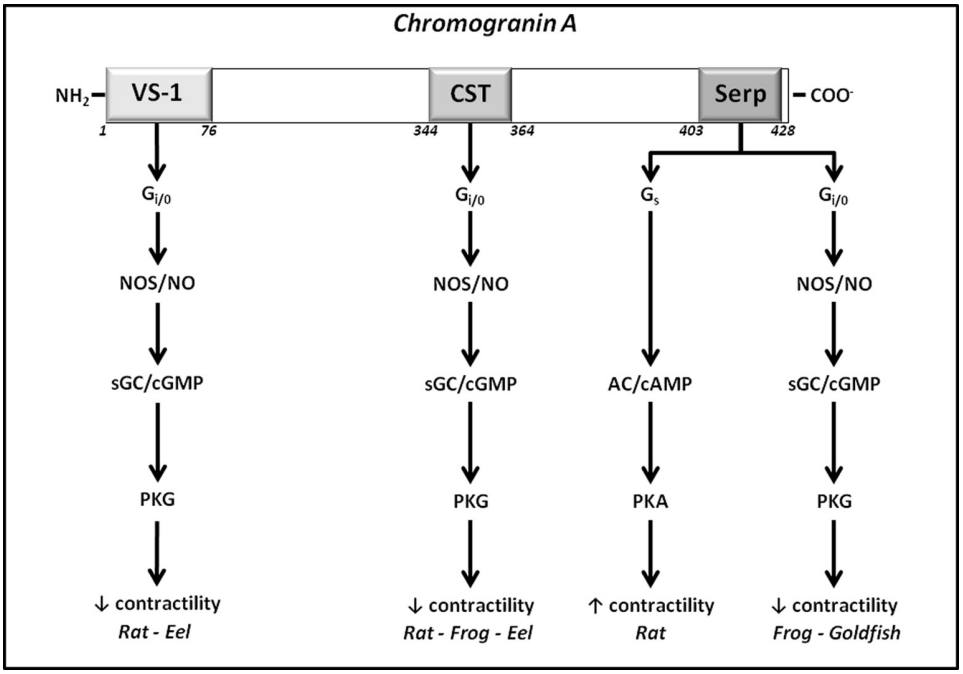

Fig. 6. Synopsis of the modulation elicited by Vasostatin-1 (VS-1), Catestatin (CST) and Serp on the heart of fish, amphibians and mammals. The involvement of the $G_{i/o}$/nitric oxide (NO)/cGMP/cGMP-dependent protein kinase (PKG) pathway in the reduction of contractility induced by VS-1 and CST (eel, frog and rat) and Serp (frog and goldfish) is illustrated. The recruitment of a cAMP/cAMP-dependent protein kinase (PKA)-dependent cascade in the increased contractility induced by Serp (rat) is also showed. Modified from Pasqua et al. (2015). sGC, soluble guanylate cyclase; AC, adenylate cyclase.

from a negative effect which is common to the various peptides (VS, CST, Serp), to a situation in which the depressing action is located in the N-terminal (VS) and the middle (CST) domains, and the stimulating action is located in the C-terminal region (serpinin) (Fig. 6). Answer to these and other questions, also using *in vivo* "animal models of stress", may contribute to clarify the role of CgA-derived peptides within the rich framework of neuroendocrine factors involved in the cardiac modulation in vertebrates.

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Conflict of interest

None.

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