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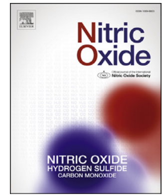

Angiotensin II dependent cardiac remodeling in the eel *Anguilla anguilla* involves the NOS/NO system

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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.

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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.

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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_{1-2}/pERK_{1-2}, 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_{1-2} (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_{1-2}

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

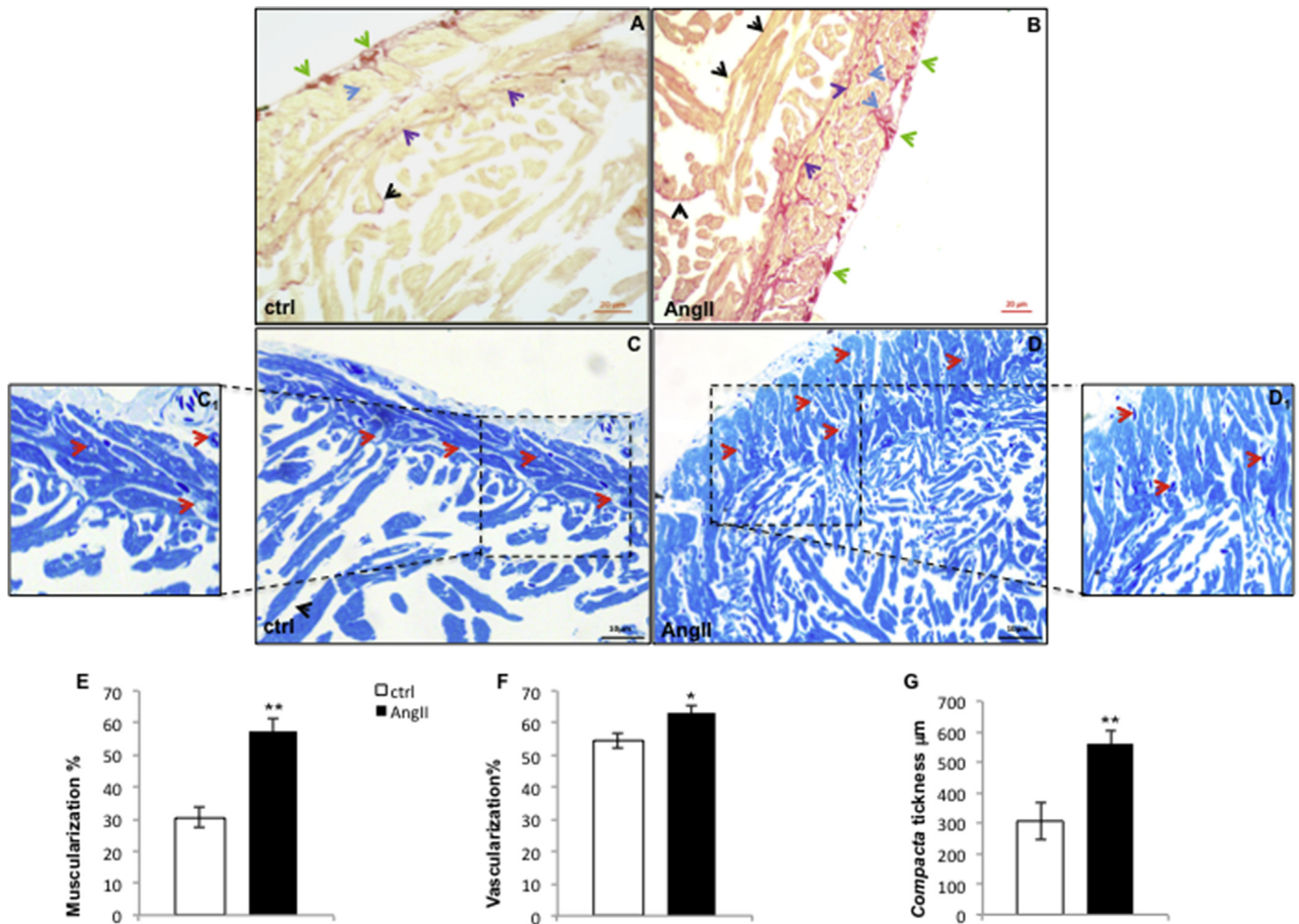

Fig. 1. Representative images showing morphological changes occurring in the eel ventricle after AngII treatment. (A, B) Sirius red staining of control (A) and AngII (B) treated hearts. Collagen fibers localize at subepicardial level (green arrows), in the vessels wall (blue arrows), at subendocardial 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

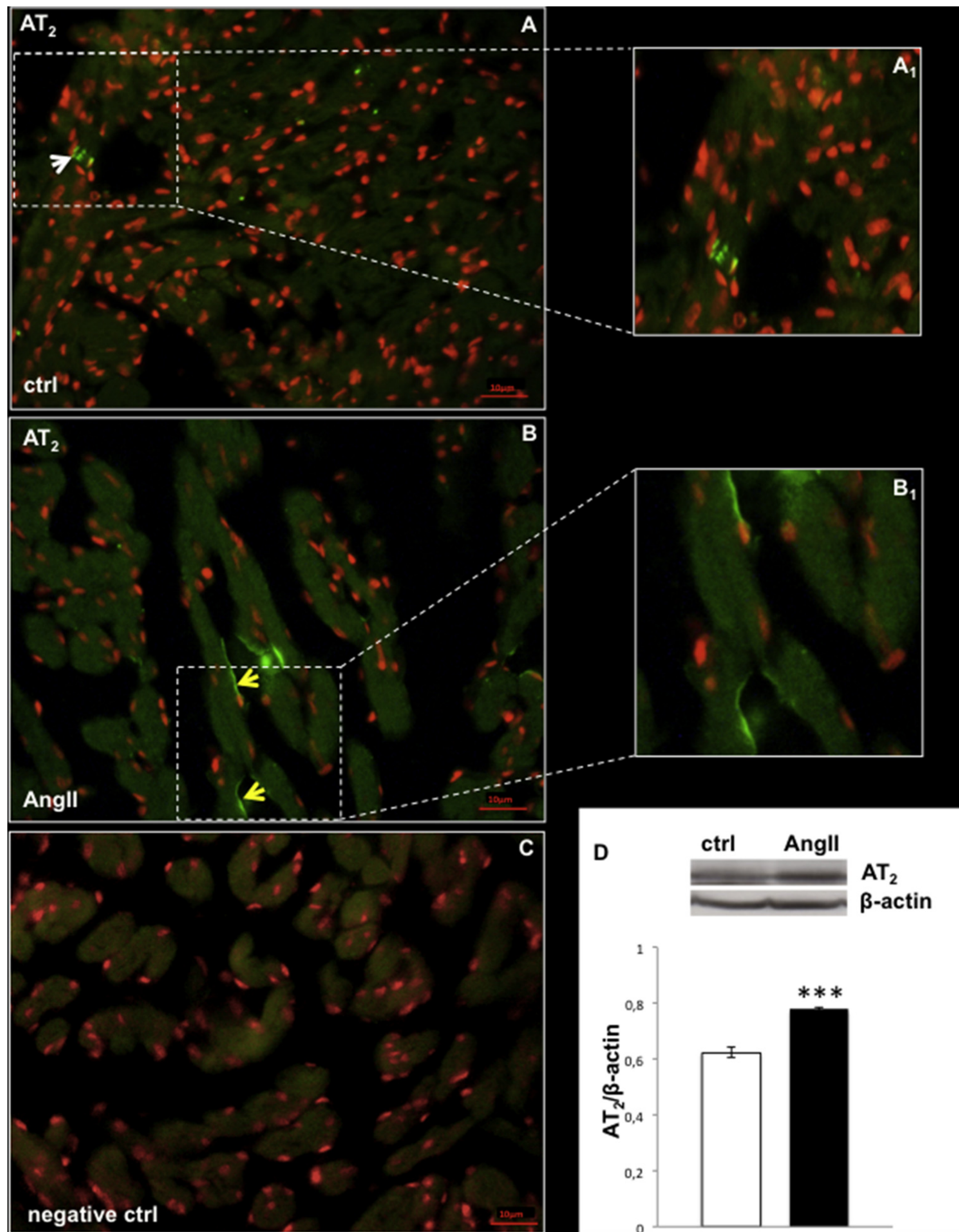

Fig. 2. (A, B) Representative images of AT_2 immunolocalization in the ventricle of control (A) and AngII treated (B) eels. White arrow: myocardiocytes. Yellow arrows: endocardial endothelium. Nuclei counterstaining: propidium iodide. Inset A_1 and B_1 represent a higher magnification of AT_2 positive 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-

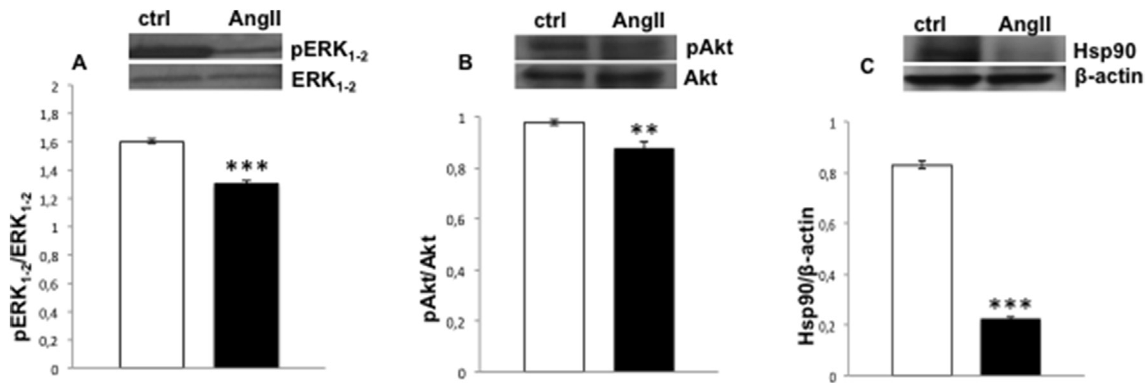

Fig. 3. Western blotting and densitometric analysis of $pERK_{1-2}/ERK_{1-2}$ (A), pAkt/Akt (B) and Hsp90 (C) in cardiac extracts of control and AngII-treated eels. In C, loaded protein amount was verified using anti-β-actin antibody. Statistical differences were evaluated by unpaired *t-test* (**p < 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 $\mu mol\ L^{-1}$ in control animals (which compares with other fish species [44,45]) to 0.6 $\mu mol\ L^{-1}$ 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

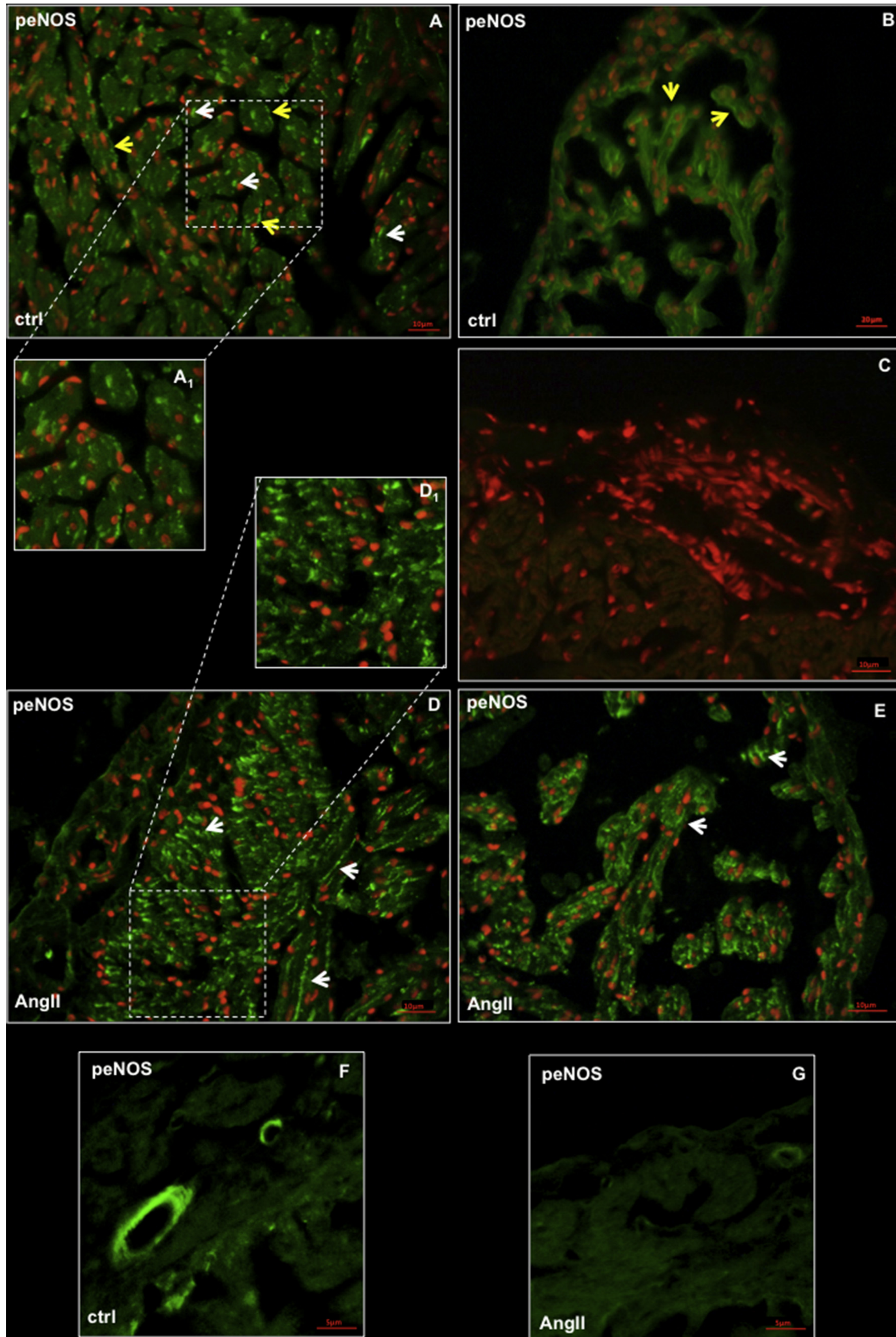

Fig. 4. Representative images showing peNOS immunolocalization on cardiac sections of control (A, B, F) and AngII (D, E, G) treated eels. (A, D) ventricle, (B, E) atrium, (F,G) vessels. Inset A_1 and D_1 represent a higher magnification of peNOS positive signal. (C) Negative control. White arrows: myocardiocytes. 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.)

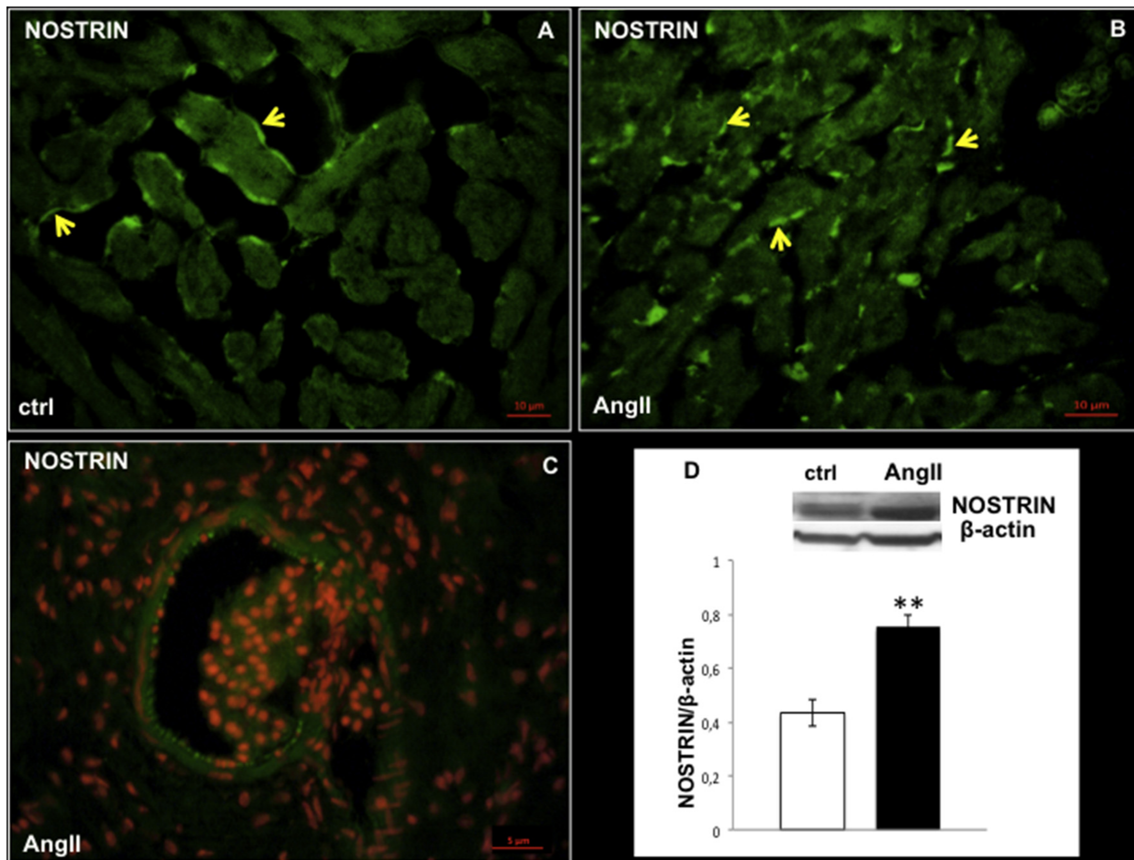

Fig. 5. Representative images of NOSTRIN immunolocalization in ventricular sections of control (A) and AngII-treated (B, C) eels. NOSTRIN localized in endocardial (yellow arrows) and vascular endothelium (red arrows). C: nuclear counterstaining with propidium iodide. (D) Western blotting of NOSTRIN in 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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