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RESEARCH ARTICLE

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

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ABSTRACT

The goldfish (*Carassius auratus*) exhibits a remarkable capacity to survive and remain active under prolonged and severe hypoxia, making it a good model for studying cardiac function when oxygen availability is a limiting factor. Under hypoxia, the goldfish heart increases its performance, representing a putative component of hypoxia tolerance; however, the underlying mechanisms have not yet been elucidated. Here, we aimed to investigate the role of β3-adrenoreceptors (ARs) in the mechanisms that modulate goldfish heart performance along with the impact of oxygen levels. By western blotting analysis, we found that the goldfish heart expresses β3-ARs, and this expression increases under hypoxia. The effects of β3-AR stimulation were analysed by using an *ex vivo* working heart preparation. Under normoxia, the β3-AR-selective agonist BRL_{37344} (10^{-12} to 10^{-7} mol l^{-1}) elicited a concentration-dependent increase of contractility that was abolished by a specific β3-AR antagonist (SR_{59230A}; 10^{-8} mol l^{-1}), but not by α/β1/β2-AR inhibitors (phentolamine, nadolol and ICI118,551; 10^{-7} mol l^{-1}). Under acute hypoxia, BRL_{37344} did not affect goldfish heart performance. However, SR_{59230A}, but not phentolamine, nadolol or ICI118,551, abolished the time-dependent enhancement of contractility that characterizes the hypoxic goldfish heart. Under both normoxia and hypoxia, adenylate cyclase and cAMP were found to be involved in the β3-AR-dependent downstream transduction pathway. In summary, we show the presence of functional β3-ARs in the goldfish heart, whose activation modulates basal performance and contributes to a hypoxia-dependent increase of contractility.

KEY WORDS: Teleost, Myocardial performance, Transduction pathway, cAMP, Adrenergic receptors

INTRODUCTION

Fluctuations in O_2 availability are common in aquatic environments. Many fish species have evolved different strategies to cope with hypoxic conditions, which allow them to balance energy supply and demand in vital organs during O_2 limitation. Among teleost fish, members of the cyprinid genus *Carassius*, such as the goldfish (*Carassius auratus*) and the crucian carp (*Carassius carassius*) exhibit a remarkable capacity to survive and remain active for long periods under hypoxia and they can even tolerate anoxia (Bickler and Buck, 2007). They show large muscle and liver glycogen reserves, a reduced metabolism and the unique ability to avoid lactic acidosis by converting, in the skeletal muscle, lactate into ethanol and CO_2, which are released through the gills (Shoubridge and Hochachka, 1980). The notable feature of these species to maintain a normal cardiac performance and autonomic cardiovascular regulation during anoxia aids in the prevention of ethanol accumulation (Stecyk et al., 2004).

In this regard, *ex vivo* studies from our laboratory documented that the isolated goldfish heart, perfused under acute hypoxia, enhances its basal performance, as well as the sensitivity to the Frank–Starling mechanism (Imbrogno et al., 2014). This may be crucial for maintaining the functional and metabolic interactions between organs and tissues under low O_2 availability. Moreover, we found that hypoxia exposure is accompanied by an increase of hypoxia inducible factor-1α (HIF-1α) and nitric oxide synthase (NOS) expression, and this supports the role of the HIF–NOS crosstalk in adjusting cardiac performance to low O_2 (Imbrogno et al., 2014). Nevertheless, the mechanisms behind the hypoxia-dependent increase of cardiac contractility in the goldfish, still remain unclear.

An attractive and putative candidate for mediating cardiac effects under hypoxic conditions is β3-adrenoreceptor (β3-AR). Since its discovery about 30 years ago, a number of studies, mainly performed in mammals, support its cardioprotective properties exerted through autocrine, paracrine and systemic effects (Balligand, 2016). Interestingly, it has been reported that β3-AR expression markedly increases in mouse B16F10 melanoma cells (Dal Monte et al., 2013a), as well as in *ex vivo* mammalian retinal explants exposed to hypoxia (Dal Monte et al., 2013b); thus suggesting an important role in the mechanisms activated under conditions of low O_2 availability.

In the heart of many teleost species, catecholamines (CAs) elicit an essentially excitatory tone, mediated by β-ARs (Ask et al., 1980; Axelsson et al., 1987; Cameron, 1979; Gamperl et al., 1994; Holmgren, 1977; Imbrogno and Cerra, 2017; Imbrogno et al., 2019). The β2-AR is believed to be the main β-AR subtype in the teleost heart, although the expression of β1-AR mRNA has been reported in the hearts of medaka and zebrafish (Kawasaki et al., 2008; Steele et al., 2011; Wang et al., 2009). Under resting and normoxic conditions, this basal adrenergic tone (% changes of R-R intervals; Altimiras et al., 1997) is generally less than the cholinergic one (Taylor et al., 2014). The adrenergic tone varies from 5 to 30% amongst species and with temperature within species, and is also complemented by circulating CAs (Taylor et al., 2014). However, with the exception of few stress-tolerant species [e.g. the members of the genus *Anguilla* (McKenzie et al., 2003) and some tropical fish (Perry et al., 2004)] it increases in response to acute physiological or environmental stress such as exercise, hypoxia, acidosis and hyperkalemia (Hanson et al., 2006; Randall and Perry, 1992; Reid et al., 1998). This allows modulation of cardiovascular and respiratory functions and recruitment of the hepatic glycogen reserves to overcome the detrimental consequences related to stressful situations.

More recently, molecular, pharmacological and physiological studies have made this picture more complex, identifying the β3-AR

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Received 29 July 2019; Accepted 11 September 2019

subtype in the heart of several teleosts, such as rainbow trout (*Oncorhynchus mykiss*; Nickerson et al., 2003; Petersen et al., 2013), European eel (*Anguilla anguilla*; Imbrogno et al., 2006), fathead minnow (*Pimephales promelas*; Giltrow et al., 2011), common carp (*Cyprinus carpio*; Petersen et al., 2015) and channel catfish (*Ictalurus punctatus*; Petersen et al., 2015). Stimulation of β3-ARs depresses stroke volume (V_S) in the eel (Imbrogno et al., 2006), rainbow trout (Petersen et al., 2013) and common carp (Petersen et al., 2015), whereas a positive inotropic effect is observed in the channel catfish (Petersen et al., 2015). The β3-AR-specific antagonist SR_{59230A} abrogates the negative effect on contractility (insensitive to the β1/β2-AR inhibitor nadolol) in the eel (Imbrogno et al., 2006) and reduced the enhancement of V_S observed in channel catfish (Petersen et al., 2015).

To the best of our knowledge, only one recent study by Motyka and colleagues analysed the correlation between hypoxia and β3-ARs in the fish heart (Motyka et al., 2017). It has been documented that the reduced cardiac pumping capacity of the hypoxia-sensitive trout (*O. mykiss*), acclimated to moderate chronic hypoxia (~40% air saturation for 17–23 weeks), correlates with a loss of cardiac β3-ARs. We therefore aimed to explore the role of the β3-AR in the modulation of the cardiac performance in a model of hypoxia-resistant teleost fish, such as the goldfish *C. auratus*, focusing on the following questions. (1) Are β3-ARs expressed in the goldfish heart? (2) Is their expression altered by acute hypoxia exposure? (3) Does the hypoxia-dependent improved pumping capacity of the goldfish heart involve β3-ARs? (4) What are the signal transduction pathways activated?

MATERIALS AND METHODS

Animals

Specimens of goldfish (*Carassius auratus* Linnaeus 1758; length, 12–16 cm; mass, 43.9±5.5 g; mean±s.e.m.) of both sexes were provided by local hatcheries. They were maintained at 18–21°C in filtered and aerated water, 12 h:12 h light:dark cycle and fed daily with commercial food. All animals were anaesthetized with MS222 (tricaine methanesulfonate; 0.2 g l^{-1}) (Sigma-Aldrich, Italy) and then killed by cervical transection; the heart was quickly dissected out and directed to the specific protocol. Animal care and experimental procedures were in accordance with Italian law (DL 27 January, 1992, no. 116) and with European Directive 2010/63/EU.

Isolated and perfused *in vitro* working heart preparations

The goldfish heart was removed without the parietal pericardium, cannulated and connected to a perfusion apparatus as previously described (Garofalo et al., 2012; Imbrogno et al., 2014). It received saline (Ringer's solution) containing (in mmol l^{-1}): NaCl 124.9, KCl 2.49, $MgSO_4$ 0.94, NaH_2PO_4 1.0, glucose 5.0, $NaHCO_3$ 15.0 and $CaCl_2$ 1.2. For normoxic experiments, saline was equilibrated with a mixture of 99.5% O_2 and 0.5% CO_2 (Imbrogno et al., 2001). For hypoxic experiments, it was equilibrated with a mixture of 10% O_2, 0.5% CO_2 and 89.5% N_2 (Imbrogno et al., 2014). pH was adjusted to 7.7–7.9. Experiments were carried out at room temperature (18–20°C). Oxygen concentrations, measured in the input reservoir by using an oxygen analyser (Milwaukee, SM600), were 8.4±0.2 mg O_2 l^{-1} (normoxia) and 2.5±0.3 mg O_2 l^{-1} (hypoxia) (means±s.e.m.), in line with those previously reported (Cameron et al., 2013; Chen et al., 2005). Hearts were stimulated with an LE 12006 stimulator (frequency identical to that of control, non-paced hearts; pulse width fixed at 0.1 ms; voltage, 1.2±0.1 V; mean±s.e.m.).

Pressures were measured with two MP-20D pressure transducers (Micron Instruments, Simi Valley, CA, USA) connected to a PowerLab data acquisition system and analysed by using Chart software (ADInstruments, Basile, Italy). Pressures were corrected for cannula resistance. Cardiac output ($\dot{Q}$) was collected over 1 min and weighed. Values were corrected for fluid density and expressed as volume measurements. Heart rate (f_H) was obtained from the periodicity of pressure traces. V_S [expressed as ($\dot{Q}/f_H$)] was used as a measure of ventricular performance. Ventricular stroke work [SW; mJ g^{-1}; (afterload−preload) V_S /ventricle mass] served as an index of systolic functionality.

Basal conditions

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

Drug application

After the 15–20 min control period, paced hearts were perfused with Ringer's solution enriched with the β3-AR-specific agonist BRL_{37344} at increasing concentrations (from 10^{-12} to 10^{-7} mol l^{-1}) to generate cumulative concentration–response curves under both normoxic and hypoxic conditions. Cardiac parameters were measured after 10 min perfusion with each drug concentration. To investigate pathways involved in the mechanism of action of β3-AR stimulation under normoxic conditions, after the stabilization, hearts were perfused according to the following protocol: (1) perfusion with BRL_{37344} (10^{-9} mol l^{-1}) for 10–15 min; (2) washout with Ringer's to return to control conditions; (3) perfusion with specific antagonist/inhibitor alone for 15–20 min; (4) perfusion with BRL_{37344} (10^{-9} mol l^{-1}) plus the specific antagonist/inhibitor for an additional 20 min. Drugs used were: the specific β3-AR antagonist SR_{59230A} (10^{-8} mol l^{-1}); a mixture of α/β1/β2-AR antagonists (phentolamine, nadolol and ICI118,551, respectively; 10^{-7} mol l^{-1}); the adenylyl cyclase inhibitor MDL-12,3330A (10^{-8} mol l^{-1}). Each experiment was completed within 2 h (Garofalo et al., 2012).

To analyse the mechanisms involved in the time-dependent enhancement of myocardial contractility observed in the goldfish heart under acute hypoxia (Imbrogno et al., 2014), after stabilization, cardiac preparations were perfused in the presence of either SR_{59230A}, or a mixture of α/β1/β2-AR antagonists or MDL-12,3330A for 60 min.

Western blotting and densitometric analysis

Western blotting analyses were used to evaluate cardiac β3-ARs expression in not-perfused goldfish hearts as well as in hearts perfused under normoxic and hypoxic conditions. Hearts were homogenized in an ice-cold homogenization buffer (250 mmol l^{-1} sucrose, 30 mmol l^{-1} Tris-HCl, 1 mmol l^{-1} EDTA, 1% SDS, pH 7.4), containing a mixture of protease inhibitors (1 mmol l^{-1} aprotinin, 20 mmol l^{-1} phenylmethylsulfonyl fluoride and 200 mmol l^{-1} sodium orthovanadate). Homogenates were centrifuged at 10,000 ***g*** for 10 min at 4°C to remove tissue debris (Rocca et al., 2018). Bradford reagent was used to determine protein concentration according to the manufacturer (Sigma-Aldrich). A 60 µg protein sample for each homogenate was separated by SDS–PAGE on 12% (w/v) polyacrylamide gels and electroblotted onto a nitrocellulose membrane (GE Healthcare). For immunodetection, blot was blocked in TBS-T (TBS with 0.2% Tween-20) containing

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5% non-fat dry milk and incubated overnight at 4°C with rabbit polyclonal antibody against β3-ARs (Santa Cruz Biotechnology, sc-50436), diluted 1:500 in TBS-T containing 1% non-fat dry milk. Protein loading for β3-AR detection was verified by using mouse β-actin (Santa Cruz Biotechnology, sc-69879). Peroxidase-linked secondary antibodies (Santa Cruz Biotechnology) were diluted 1:1000 in TBS-T containing 5% non-fat dry milk and incubated for 1 h at room temperature. Immunodetection was performed by using an enhanced chemiluminescence kit (ECL PLUS, GE Healthcare). Autoradiographs were scanned to obtain arbitrary densitometric units. Experiments were performed in triplicate and the results expressed as mean±s.e.m. of absolute values.

cAMP determination

cAMP levels were measured in homogenates from normoxic, normoxic plus BRL_{37344} and hypoxic perfused goldfish hearts. Samples were treated with 5% trichloroacetic acid on ice and centrifuged at 1500 ***g*** for 10 min. The supernatant was extracted three times with 5 volumes of diethyl ether saturated with water, and the aqueous phase was collected and used for cAMP measurements by using a commercial enzyme immunoassay (cAMP ELISA Kit; Cayman Chemical).

Drugs and chemicals

BRL_{37344}, SR_{59230A}, phentolamine, nadolol, ICI118,551 and MDL-12,3330A were purchased from Sigma-Aldrich. Phentolamine, nadolol and ICI118,551 were prepared in double-distilled water. BRL_{37344}, SR_{59230A} and MDL-12,3330A were dissolved in DMSO (maximum final concentration less than 0.1%). At this concentration, DMSO alone was found to have no effect on cardiac performance (data not shown). All dilutions were made in Ringer's solution immediately before use.

Statistics

For physiological experiments, data were expressed as means±s.e.m. of percentage changes obtained from individual experiments. Statistical analysis was determined by using unpaired *t*-test, or one-way ANOVA, or repeated measures ANOVA followed by Bonferroni's or Dunnett's post-test. Differences were considered statistically significant at $*P<0.05$. For densitometric analyses and cAMP determination, values were expressed as means±s.e.m. of absolute values from individual experiments; statistic was assessed by unpaired *t*-test. Significance was concluded at $*P<0.05$. GraphPad Prism software, v.4.02 (GraphPad Software Inc., San Diego, CA, USA) was used for the statistical analysis.

RESULTS

Cardiac β3-AR expression

Basal cardiac β3-AR expression was evaluated in homogenates from non-perfused and perfused hearts under both normoxic and hypoxic conditions. Western blot analysis revealed an immunoreactive band corresponding to the approximate molecular mass of β3-AR (44 kDa) in cardiac extracts from all experimental conditions. With respect to both non-perfused hearts and hearts perfused under normoxic conditions, a significant increase of β3-AR expression was observed following acute hypoxia (Fig. 1).

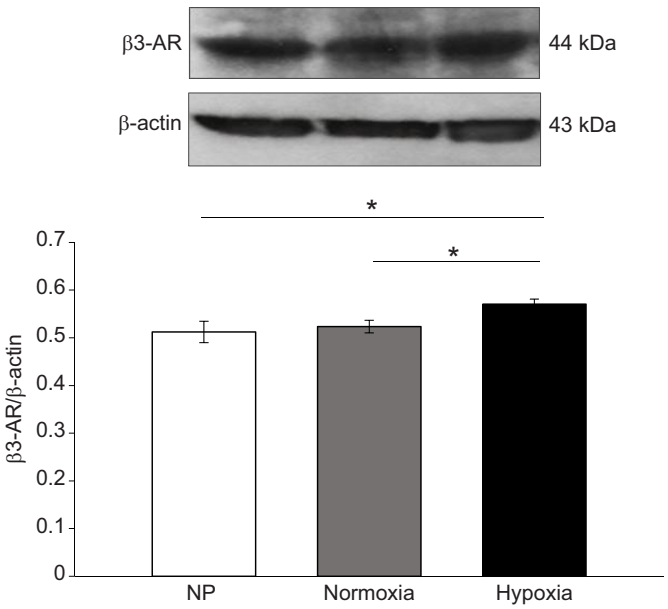

Fig. 1. Cardiac β3-AR expression in the goldfish heart. β3-AR protein levels (relative to that of β-actin) in non-perfused (NP) and perfused hearts under either normoxia or hypoxia. Significance of differences from control values (unpaired *t*-test): $*P<0.05$. Values are means±s.e.m. of three experiments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

Isolated heart preparation

After stabilization, the *ex vivo* isolated and perfused goldfish heart preparation values of preload, afterload, f_H, $\dot{Q}$, V_S and SW (Table 1) were comparable to those previously reported [normoxia (Imbrogno et al., 2017; Mazza et al., 2015, 2019); hypoxia (Imbrogno et al., 2014; Mazza et al., 2019)].

Effects of β3-AR stimulation under normoxia

Dose–response curve of BRL_{37344}

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

Effects of BRL_{37344} after treatment with adrenergic antagonists

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

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

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

$\dot{Q}$, cardiac output; V_S, stroke volume; f_H, heart rate; SW, stroke work. Values are means±s.e.m. of $N=19$ for normoxia and $N=15$ for hypoxia.

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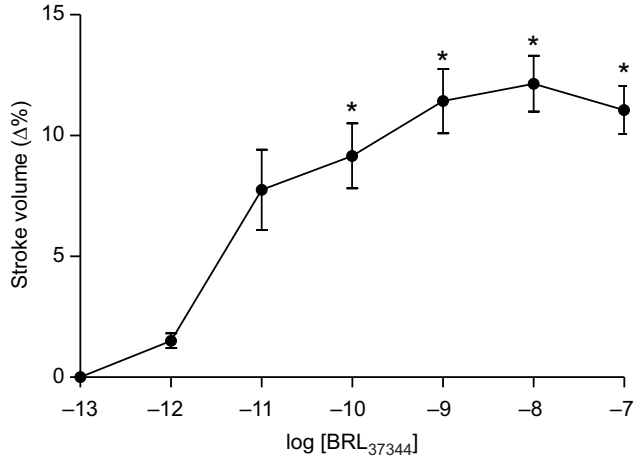

Fig. 2. Cumulative dose–response curve of BRL_{37344} on stroke volume in isolated and perfused working goldfish heart. Percentage changes were evaluated as means±s.e.m. of 6 experiments. Significance of difference from control values (repeated measures ANOVA; Dunnett's *post hoc* test): **P*<0.05. *x*-axis shows log of BRL_{37344} concentration in mol l^{-1}.

Involvement of the adenylate cyclase/cAMP signal transduction pathway

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

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

Dose-response curve of BRL_{37344}

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

Effects of adrenergic antagonists

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

Involvement of the adenylate cyclase/cAMP signal transduction pathway

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

DISCUSSION

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

β3-AR expression in the normoxic and hypoxic goldfish heart

In mammals, the heart is a major β3-AR-expressing organ. The receptor localizes on both the myocardium and the coronary endothelium (Dessy et al., 2004), and plays a role in cardiac function and remodelling (Balligand, 2016; Imbrogno et al., 2015). Currently, very few studies document the presence of β3-ARs in the teleost heart. In 2003, Nickerson and co-workers detected two β-ARs in *O. mykiss* (adrb3a, NP_001118100; adrb3b, NP_00117924) (Nickerson et al., 2003). These β-ARs are homologous to the mammalian β3-AR and are highly expressed in the heart. Subsequently, sequences similar to β3-ARs were identified in various teleost species, including zebrafish (adrb3a: BAH84778 and adrb3b: NP_001128606), black bullhead (adrb3b: ABH10580), stickleback (ENSGACP00000014582) and fugu (ENSTRUP 00000020 757) (for references, see Imbrogno et al., 2015).

In the present study, we found that β3-ARs are expressed in the goldfish heart. A significant increased expression was observed in hearts exposed to hypoxia compared with both non-perfused hearts and hearts perfused under normoxia. These results are in line with the upregulation of β3-ARs observed in *ex vivo* mouse retinal explants exposed to low O_2 (Dal Monte et al., 2013b). However, they conflict

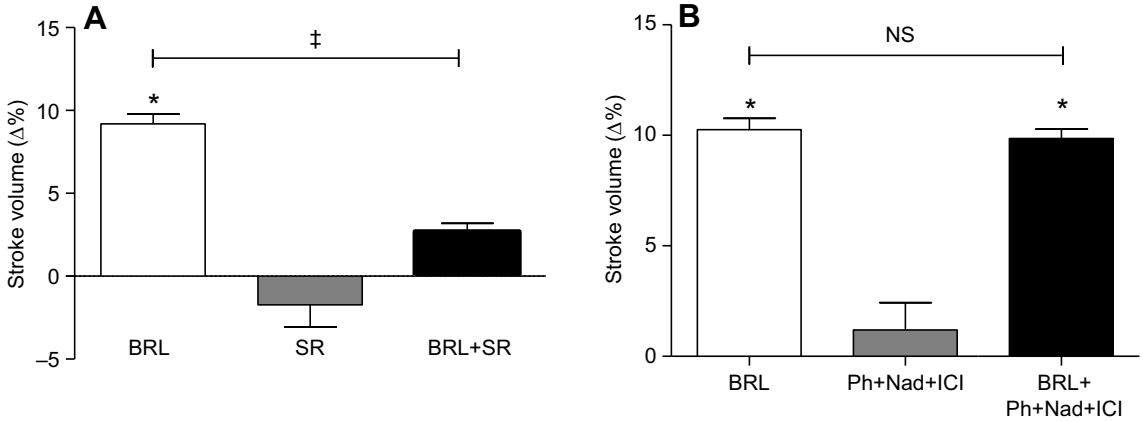

Fig. 3. Effects of BRL_{37344} on stroke volume of isolated and perfused working goldfish hearts before and after treatment with antagonists. BRL_{37344} (10^{-9} mol l^{-1}) effects before and after treatment with (A) SR_{59230A} (10^{-8} mol l^{-1}) and (B) a mixture of α/β1/β2 antagonists (10^{-7}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

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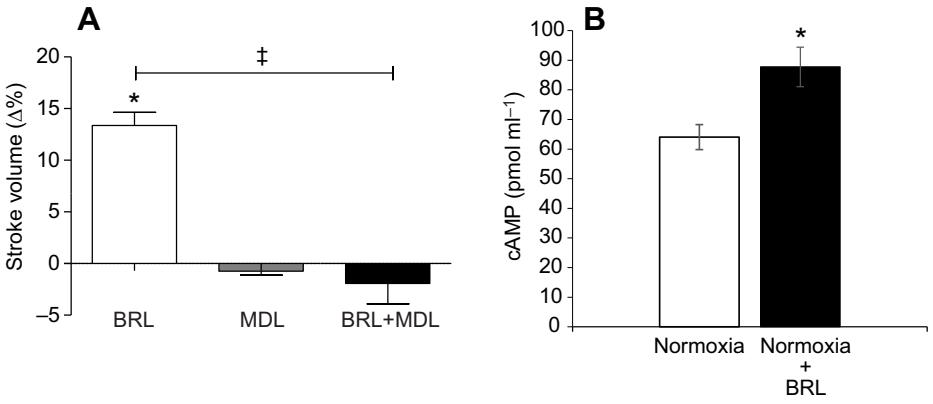

Fig. 4. Involvement of the AC/cAMP pathway in the BRL37344-dependent increase of contractility in the isolated and perfused goldfish heart. (A) Effects of BRL_{37344} (10^{-9} mol l^{-1}) on stroke volume before and after treatment with MDL-12,3330A (10^{-8} mol l^{-1}). Percentage changes were evaluated as means± s.e.m. of 4 experiments. Differences are indicated as: **P*<0.05 (BRL versus control), ‡*P*<0.05 (BRL versus BRL+inhibitor); repeated measures ANOVA followed by Bonferroni's *post hoc* test. (B) cAMP levels in goldfish cardiac extracts under normoxia with and without BRL_{37344} treatment. Significance of difference from control values (unpaired *t*-test): **P*<0.05 (*n*=3). BRL, BRL_{37344}; MDL, MDL-12,3330A.

with observations in the hypoxia-sensitive trout heart, where the hypoxia-dependent reduced pumping capacity has been attributed to a loss of cardiac β3-ARs (Motyka et al., 2017). It cannot be excluded that in fish the influence of hypoxia on cardiac β3-AR expression correlates with the species-specific ability to face low oxygen (e.g. hypoxia-sensitive trout versus hypoxia-tolerant goldfish).

Effects of β3-AR stimulation in the normoxic goldfish heart

In the isolated goldfish heart perfused under normoxic conditions, β3-AR stimulation by BRL_{37344} induced a concentration-dependent increase of contractility. This effect was abolished by the β3-AR-specific antagonist SR_{59230A}, which acts in a competitive manner in teleosts (Imbrogno et al., 2015). In contrast, it was not modified by a mixture of α/β1/β2-AR antagonists (phentolamine, nadolol and ICI118,551, respectively), free of β3-ARs antagonist properties (Emorine et al., 1989; Galitzky et al., 1993). This excluded the involvement of these AR types in the hemodynamic effects elicited by β3-AR activation.

In mammals, a promiscuous coupling of β3-ARs to either $G_{i/o}$ or G_s proteins has been proposed to describe the different cardiac effects so far reported (Imbrogno et al., 2015; Sterin-Borda et al., 2006). As originally proposed by Gauthier et al. (1998, 1996), and then confirmed by several experimental evidences, cardiac β3-ARs are generally considered to be coupled to $G_{i/o}$ and to a transduction mechanism that, through the involvement of nitric oxide (NO), leads to a reduction of Ca^{2+} transients (Kitamura et al., 2000; Mazza et al., 2010) and dampens the stimulatory effects induced by cAMP cascades. In parallel, several lines of evidence suggest a role for G_s proteins (Bardou et al., 2000; Mattsson et al., 2010; Zhang et al., 2012). This is the case for the positive inotropism observed in the mouse heart after stimulation of β3-ARs that is associated with a G_s-dependent activation of AC and the consequent cAMP generation (Kohout et al., 2001). A G_s-induced increase of L-type Ca^{2+} channel current ($I_{Ca,L}$), through a cAMP/PKA mechanism, has been also used to explain the increased contractility induced by β3-AR agonists SR_{59230A}, BRL_{37344} and CGP_{12177} in human atrial myocytes (Skeberdis et al., 2008).

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

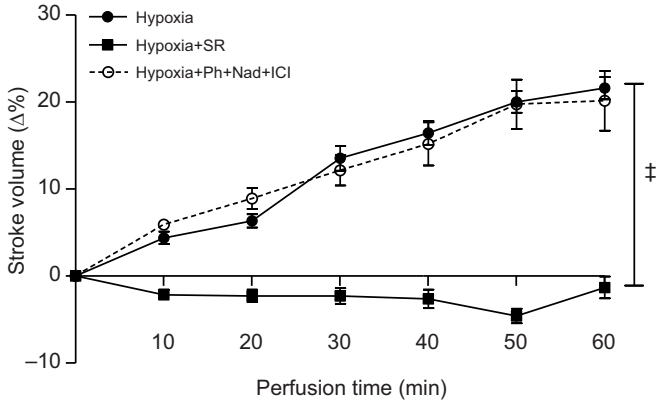

Fig. 5. Effects of AR antagonists on the hypoxia-induced increase of contractility in the isolated and perfused goldfish heart. Time-course curves of stroke volume before and after treatment with SR_{59230A} (10^{-8} mol l^{-1}) and with a mixture of α/β1/β2 AR antagonists (10^{-7} mol l^{-1}). Data are expressed as means±s.e.m. of 4 experiments for each group. Differences are indicated as: ‡*P*<0.05; one-way ANOVA followed by Bonferroni's *post hoc* test. ICI, ICI118,551; Nad, nadolol; Ph, phentolamine; SR, SR_{59230A}.

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

A remarkable feature of the goldfish heart exposed to acute hypoxia is its ability to enhance the basal cardiac performance and the sensitivity to the Frank–Starling mechanism (Imbrogno et al., 2014). This has been considered to be an important mechanism for maintaining functional and metabolic interactions between organs and tissues, required for the hypoxia tolerance of the whole organism (Gattuso et al., 2018). We observed that under acute administration of moderate hypoxia, β3-AR-selective inhibition by SR_{59230A} abolished the time-dependent improved cardiac performance of the hypoxic goldfish heart. This effect was not observed by administration of phentolamine (a non-selective α-AR antagonist), nadolol (a non-selective β-AR antagonist) and ICI118,551 (a selective β2-AR antagonist), thus ruling out the involvement of α/β1/β2-ARs. Unlike normoxia, under hypoxia, β3-AR activation by BRL_{37344} did not affect goldfish heart performance. This result is intriguing but deserves further study to establish whether in the goldfish differences in oxygen availability affect myocardial β3-AR sensitivity.

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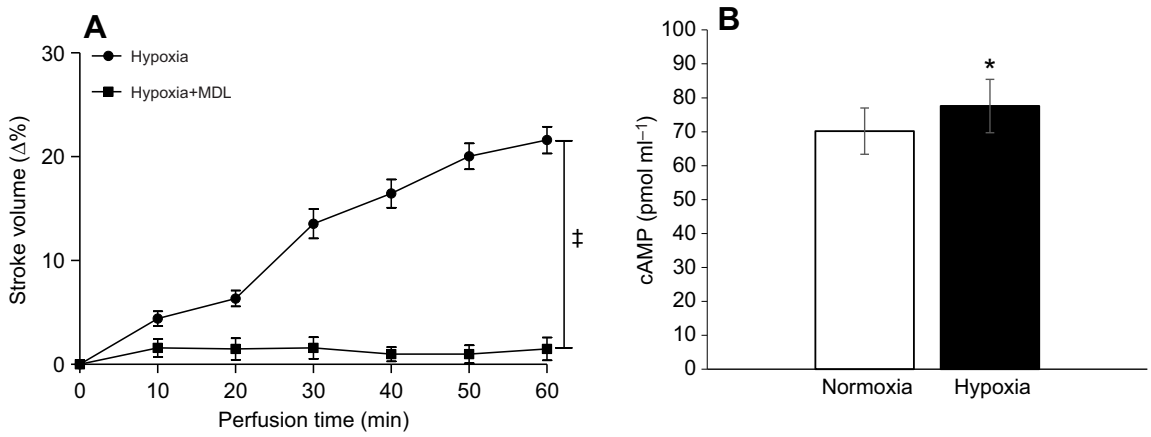

Fig. 6. Involvement of the AC/cAMP pathway in the hypoxia-dependent increase of contractility in the isolated and perfused goldfish heart. (A) Time-course curves of stroke volume before and after treatment with MDL-12,3330A (10^{-8} mol l^{-1}). Data are expressed as mean values±s.e.m. of 3 experiments.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

The study of the mechanism of action elicited by β3-AR activation showed that, as in normoxia, the AC–cAMP cascade is involved. This was suggested by the effect induced by the AC inhibitor MDL-12,3330A on the time-course experiments, and by the increased levels of cAMP we observed in the hypoxia-exposed hearts.

Currently, the specific mechanism responsible for β3-AR activation in our isolated hypoxic goldfish hearts is unknown. Interestingly, as reported by Newton and co-workers, a rich adrenergic innervation, which may allow a fine autonomic control of the cardiac function, characterizes the goldfish heart (Newton et al., 2014). Moreover, it has been proposed that the isolated and perfused goldfish heart is able to release CAs, possibly from intracardiac chromaffin tissues (Cameron and O'Connor, 1979), and that in the hypoxia-tolerant tropical fish red-bellied piranha (*Pygocentrus nattereri*), an endogenous release of cardiac CAs is able to rescue myocardial performance during hypoxia, even in the absence of humoral CAs (Joyce et al., 2019). In this context, the possibility that in the goldfish heart, perfused under hypoxia, β3-ARs may be activated by CAs released by either endogenous chromaffin tissues and/or by nerve terminals, should be considered.

Conclusions

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

Acknowledgements

Authors thank Prof. Verri Tiziano from the University of Salento (Lecce, Italy) for his support with the hemodynamic study.

Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: S.I.; Methodology: S.L., A.G., R.M., M.F.; Formal analysis: R.M., M.F.; Investigation: M.F.; Data curation: S.L., A.G., R.M., M.F.; Writing - original draft: S.I.; Writing - review & editing: S.L., A.G., M.C.C., S.I.; Supervision: M.C.C., S.I.; Project administration: S.I.

Funding

This work was supported by MIUR (Ministero dell'Istruzione, dell'Università e della Ricerca) of Italy, 'Dottorato di Ricerca in Life Science and Technology', University of Calabria.

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