Journal of Molecular and Cellular Cardiology 108 (2017) 95–105

Contents lists available at ScienceDirect

Journal of Molecular and Cellular Cardiology

journal homepage: www.elsevier.com/locate/yjmcc

Induction of cardiac dysfunction in developing and adult zebrafish by chronic isoproterenol stimulation

Mandy Kossack [a,b], Selina Hein [a,b], Lonny Juergensen [a], Mauro Siragusa [a,b,1], Alexander Benz [a,b], Hugo A. Katus [a,b], Patrick Most [a,b,c,**], David Hassel [a,b,*]

[a] Department of Medicine III, Cardiology, Angiology, Pneumology, University Hospital Heidelberg, INF 410, 69120 Heidelberg, Germany
[b] DZHK (German Centre for Cardiovascular Research), Partner Site Heidelberg/Mannheim, INF 669, 69120 Heidelberg, Germany
[c] Center for Translational Medicine, Department of Medicine, Thomas Jefferson University, 1020 Walnut Street, Philadelphia, Pennsylvania, USA

ARTICLE INFO

Article history:
Received 7 April 2017
Received in revised form 24 May 2017
Accepted 26 May 2017
Available online 26 May 2017

Keywords:
Zebrafish
Beta-adrenergic
Heart failure
Animal model
Isoproterenol

ABSTRACT

Zebrafish is a widely used model to evaluate genetic variants and modifiers that can cause heart muscle diseases. Surprisingly, the β-adrenergic receptor (β-AR) pathway in zebrafish is not well characterized, although abnormal β-AR signaling is a major contributor to human heart failure (HF). Chronic β-AR activation in the attempt to normalize heart function in the failing heart results in a reduction of the β-ARs expression and receptor desensitization, largely mediated through G-protein coupled receptor kinase 2 (GRK2) upregulation. This in turn leads to further deterioration of heart function and progression towards HF. This study seeks to systematically characterize the function of the β-AR signaling in developing and adult zebrafish to ultimately assess the ability to induce HF through chronic β-AR activation by isoproterenol (ISO) as established in the mouse model.

Larval hearts first responded to ISO by 3 dpf, in concordance with robust expression of key components of the β-AR signaling pathway. Although ISO-induced β1-AR and β2-AR isoform upregulation persisted, chronic ISO stimulation for 5 d caused systolic cardiac dysfunction concurrently with maximal expression of G-protein-coupled receptor kinase-2 (GRK2). More consistent to mammalians, adult zebrafish developed significant heart failure in concert with β1-AR downregulation, and GRK2 and brain natriuretic peptide (BNP) upregulation in response to prolonged, 14 d ISO-stimulation. This was accompanied by significant cell death and inflammation without detectable fibrosis. Our study unveils important characteristics of larvae and adult zebrafish hearts pertaining to β-AR signaling. A lack of β-AR responsiveness and atypical β-AR/GRK2 ratios in larval zebrafish should be considered. Adult zebrafish resembled the mammalian situation on the functional and molecular level more closely, but also revealed differences to dysfunctional mammalian hearts, i.e. lack of fibrosis. Our study establishes the first ISO-inducible HF model in adult zebrafish and present critical characteristics of the zebrafish heart essential to be considered when utilizing the zebrafish as a human disease and future drug discovery model.

1. Introduction

Over the past decades the zebrafish became a widely used cardiac disease model [1]. Large whole genome mutagenesis screens and modern reverse genetics approaches in zebrafish identified hundreds of new potential cardiac disease genes, genetic variants and modifiers causative for human heart muscle diseases [2,3]. Recently, larval zebrafish are used for high throughput therapeutic molecule testing and identification of novel cardio-protective targets [4–6]. Although abnormal β-adrenergic receptor signaling is common in human heart disease and contributes significantly to cardiac failure, the role of β-adrenergic receptor (β-AR) signaling and its contribution to cardiovascular pathologies in zebrafish is not known.

During heart failure (HF) in humans, the sympathetic nervous system is activated to normalize cardiac output. While being harmless when released transiently, chronic β-AR activation by the catecholamines triggers maladaptive changes in the diseased heart, associated with β-AR desensitization due to receptor downregulation and thus reduction of catecholamine responsiveness of the heart that ultimately results in a further decline in cardiac contractility and in the progression towards HF [7]. Chronic β-AR activation alone can induce HF. Long-term

Abbreviations: β-AR, beta-adrenergic receptor; GRK2, G-protein-coupled receptor kinase-2; ISO, isoproterenol; HF, heart failure; PKA, protein kinase A; ANP, atrial natriuretic peptide; BNP, brain natriuretic peptide; FS, fractional shortening; HR, heart rate; PRO, propranolol; TH, tyrosine hydroxylase; CHX, cycloheximide.

* Correspondence to: David Hassel, University Hospital Heidelberg, Department of Internal Medicine III, Cardiology, Im Analysezentrum 669, 69120 Heidelberg, Germany.
** Correspondence to: Patrick Most, University Hospital Heidelberg, Department of Internal Medicine III, Cardiology, Im Neuenheimer Feld 410, 69120 Heidelberg, Germany.
E-mail addresses: patrick.most@med.uni-heidelberg.de (P. Most), david.hassel@med.uni-heidelberg.de (D. Hassel).
[1] Present address: Institute for Vascular Signalling, Centre for Molecular Medicine Goethe University,Theodor Stern-Kai 7, 60590 Frankfurt, Germany.

http://dx.doi.org/10.1016/j.yjmcc.2017.05.011
0022-2828/

Isoproterenol (ISO) treatment in mice represents a well-established HF model to investigate novel mechanisms and test new therapeutic strategies [8–13]. Standard treatment for patients with HF includes the administration of β-blockers in an attempt to counteract desensitization and thus to improve survival and slow disease progression [14]. The G-protein-coupled receptor kinase (GRK2) plays a crucial role in mediating the β-AR desensitization and hence the progression of HF. GRK2 is consistently upregulated in HF in various animal models and in humans. Therapeutic blockage of GRK2 activity demonstrated superior beneficial effects as compared to standard therapies and significantly improved functional and morphological parameters of the failing heart [15–18].

Here, we seeked to systematically analyze the function of the β-AR system in the developing larval and in the adult zebrafish heart and its contribution to cardiac failure when chronically activated. Our study uncovered important functional and disease relevant characteristics of the β-AR signaling comparing larval and adult zebrafish hearts. Stage dependent lack of β-AR responsiveness and model specific expression changes of β-AR isoforms during GRK2 dependent desensitization have to be considered particularly for future therapeutic screens in zebrafish larvae within the first three days. The adult zebrafish however closely resemble the mammalian situation, thus representing the first ISO-inducible heart failure model in adult zebrafish.

2. Materials and methods

All animal experiments were performed in accordance with the guidelines of the state of Baden-Wuerttemberg and were approved by the Regierungspräsidium Karlsruhe.

2.1. Isoproterenol exposure

All isoproterenol (ISO, Sigma-Aldrich, München, Germany) solutions were freshly prepared in water or diluted directly in the media of the zebrafish to the respective final concentration. Tissue ISO availability was not measured specifically, but ISO dosage was determined based on the biological effect induced. To guarantee a constant ISO-concentration, the media, substituted with the respective amount of ISO, was changed daily. For evaluation of cardiac performance, zebrafish embryos (strain *AB* or *Tg[myl7:gfp]*) were embedded in methylcellulose (warmed up to room temperature) and videos of the heart were recorded with a Carl Zeiss video microscope. To calculate the heart frequency, the heart beat was counted for 10 sec and extrapolated to heart beat. To calculate the fractional shortening, the diameter of the ventricle during diastole and systole were measured. Subsequently, fractional shortening was calculated using the commonly used equation $V_{diastole} - V_{systole} / V_{diastole} \times 100$ ($V_{diastole}$, diastolic ventricular diameter; $V_{systole}$, systolic ventricular diameter). The measurements of the cardiac function were performed at room temperature in larval and adult zebrafish.

After ISO-exposure, zebrafish were immediately transferred to 200-300 mg/l Tricainemethansulfunate (Sigma-Aldrich, München, Germany) solution for heart explantation. For whole heart imaging, the hearts were directly transferred in PBS and imaged using a stereoscopic microscope (SZX16, Olympus, Hamburg, Germany).

2.2. Quantitative real time-PCR

Quantitative RT–PCR (qPCR) was carried out using RNA extracted from whole embryos, embryo hearts or adult heart tissue using Trizol reagent (QIAzol Lysis Reagent, Qiagen, Hilden, Germany) and reverse transcribed with the iScript cDNA synthesis kit (Bio-Rad, München, Germany). The transcription levels of target genes were measured using gene-specific primers (Supplementary Table S1) and SYBR Green reagent (QuantiFast SYBR Green PCR Kit, Qiagen, München, Germany). The $2^{-\Delta\Delta Ct}$ method was utilized to normalize the gene of interest to the endogenous housekeeping gene *EF1α* and to determine the fold change relative to controls.

2.3. Echocardiography

For echocardiographic examinations, 6-12 months old adult zebrafish were anesthetized applying 3.2 μM 2-Phenoxyethanol to the maintenance water and analyzed with the Vevo2100 Imaging system (Visual Sonics, Amsterdam, Netherlands) [19,20].

Echocardiography in adult zebrafish was performed as described recently [19,20]. In brief, the Vevo2100 Imaging system equipped with a high frequency transducer (MS700, 30-70 MHz) was utilized to examine adult zebrafish sedated with 2-Phenoxyethanol (Sigma-Aldrich, München, Germany). Two cardiac examination planes were recorded: a long axis view and an abdominal-cranial axis to attain pulse-waved Doppler measurements. We calculated fractional shortening, ejection fraction and stroke volume by using the long axis view. Applying the abdominal-cranial axis we analyzed the heart rate.

2.4. PKA assay

PKA activity was measured using the PepTag non-radioactive cAMP-dependent protein kinase assay (Promega, Mannheim, Germany) according to manufacturer's instructions. Zebrafish hearts were dissected and treated with ISO *in vitro* in Tyrode´s solution. Treated and control adult zebrafish hearts were snap-frozen in liquid nitrogen. The assays of PKA activity rely on a change in charge of the fluorescent PepTag® A1 peptides from +1 to −1 following phosphorylation. Bands were visualized using UV light and the ratio of fluorescence intensity of phosphorylated to non-phosphorylated peptide was quantified using Image Lab software (BioRad, Version 4.0.1).

2.5. TUNEL and AFOG staining

For histological analyses hearts were embedded in Tissue-Tek O.C.T.™ Compound (Sakura, Staufen, Germany) and stored at −80 °C. Cryosections were cut with a cryostat (10 μm thickness, Microm HM560; Thermo Scientific Microm, Walldorf, Germany) at −20 °C and transferred to SuperFrost Plus slides (Thermo Scientific, Karlsruhe, Germany). Cell death was analyzed in cryosections from control or ISO-stimulated zebrafish hearts by TdT-mediated dUTP-biotin nick end labeling (TUNEL) staining (In Situ Cell Death Detection Kit TMR red, Fluorescein, Roche Applied Science, Mannheim, Germany), following the manufacturer's instructions. Nuclei were counterstained with DAPI (4′,6-Diamidino-2-Phenylindole, Dihydrochloride, Life Technology, Carlsbad, USA). Sections were then mounted with FluorSave™ (Calbiochem, Darmstadt, Germany) and imaged using a Carl Zeiss fluorescence microscope. Acid Fuchsin Orange-G (AFOG) staining was performed as described previously [21].

2.6. Isolation of cardiomyocytes and measurement of calcium transients

Adult cardiomyocytes were isolated from untreated and ISO-treated wildtype zebrafish according to Sander et al. [22]. Briefly, isolated hearts were covered with digestion solution containing collagenase 2 and collagenase 4 and incubated for 2 h at 32 °C under continuous shaking. After centrifugation, supernatant was removed and pellets were carefully resuspended in plating medium (MEM plus 2 mM Glutamin, 5 mM BDM, 5 % (vol/vol) FBS, 100 U ml -1 penicillin-streptomycin). Cardiomyocytes were seeded into tissue culture dishes with cover glass bottom (World Precision Intruments, Herts, UK) coated with 10 μg/mL laminin (Sigma-Aldrich, München, Germany). Calcium transients were assessed in Fura-2, AM field-stimulated cardiomyocytes employing epifluorescent digitalized microscopy, as follows: adherent cardiomyocytes were incubated for 20 min with 1 μM Fura-2, AM (Life Technologies, Carlsbad, USA) and then washed 3 times with medium. Fluorescence measurements were carried out using an inverse Olympus microscope (IX70) with a UV filter connected to a monochromator (Polychrome II, TILL Photonics GmbH, Germany). Cells were electrically stimulated with 1 Hz and excited at 340/380 nm. Fluorescence emission was detected at 510 nm, digitized,