DYRK2 Negatively Regulates Cardiomyocyte Growth by Mediating Repressor Function of GSK-3β on eIF2Bε

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Abstract

Background: A prerequisite of hypertrophic response of the myocardium is an increase in protein synthesis. A central regulator of translation initiation is Eukaryotic initiation factor 2B (eIF2B). Here we assessed the hypothesis that regulation of protein synthesis via eIF2Bε is essential to cardiac hypertrophic response in vivo.

Methods: Two transgenic mouse lines were generated with cardiac restricted overexpression of eIF2Bε or its mutant eIF2Bε-eIF5S35A, which cannot be inactivated by phosphorylation through GSK-3β.

Results: (1) Under baseline conditions eIF2Bε transgenic mice showed no difference in cardiac phenotype compared to wild type, whereas in the mutant eIF2Bε-S35A an increase in LV/tibia length (7.5±0.4 mg/mm vs. 6.2±0.2 mg/mm, p<0.001) and cardiomyocyte cross sectional area (13004±570 vs. 10843±347 RU, p<0.01) was observed. (2) Cardiac overexpression of eIF2Bε did not change the response of the heart to pathologic stress induced by chronic isoproterenol treatment. (3) Cardiac overexpression of the eIF2Bε transgene was followed by overexpression of DYRK2 which is known to prime the inhibitory action of GSK-3β on eIF2Bε, while DYRK1A and GSK-3β itself were not increased. (4) In C57BL/6 mice after 48 h of isoproterenol-stimulation or aortic banding, eIF2Bε was increased and DYRK2 was concomitantly decreased. (5) In line with these in vivo findings, siRNA knockdown of DYRK2 in cultured cardiomyocytes resulted in decreased levels of p(S535)-eIF2Bε, whereas adenoviral induced overexpression of DYRK2 was accompanied by clearly increased phosphorylation of eIF2Bε, indicating a coordinated response pattern. (6) Adenoviral induced overexpression of DYRK2 leads to significantly reduced cardiomyocyte size and diminishes hypertrophic response to adrenergic stimulation.

Conclusions: The interaction of GSK-3β and its priming kinase DYRK2 regulate the activity of eIF2Bε in cardiac myocytes. DYRK2 is a novel negative regulator of cardiomyocyte growth. DYRK2 could serve as a therapeutic option to regulate myocardial growth.

Introduction

Left ventricular hypertrophy (LVH) is an important risk factor for ischemia, arrhythmia and sudden death, independent of the underlying hypertrophic stimulus [1]. Given the limited ability of current pharmacological principles to prevent or regress LVH, novel therapeutic strategies are urgently needed to prevent LVH and its progression rather than to treat established cardiomyopathy and heart failure. The hypertrophic growth of the heart results primarily from an increase in cardiomyocyte size and not from proliferation [2]. Hence, the regulation of overall rates of protein synthesis in cardiomyocytes is the primary determinant for the growth associated with LVH. Anyhow, the molecular mechanisms by which protein synthesis and cardiac growth is regulated have received little attention so far.

We have previously shown that the guanine nucleotide exchange factor 2B is one of these crucial regulators of protein synthesis and cardiomyocyte growth in vitro [3]. eIF2B is a guanine nucleotide exchange factor which mediates the exchange of GDP bound to translation initiation factor eIF2 for GTP. This exchange process is a key regulatory step for the control of translation initiation in all eukaryotic organisms and increases thereby protein synthesis. eIF2B is composed of five subunits termed α-ε in order of increasing size [4]. The 82kDa ε-subunit is

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Citation: Weiss CS, Ochs MM, Hagenmueller M, Streit MR, Malekar P, et al. (2013) DYRK2 Negatively Regulates Cardiomyocyte Growth by Mediating Repressor Function of GSK-3β on eIF2Bε. PLoS ONE 8(9): e70848. doi:10.1371/journal.pone.0070848

Editor: Rossella Rota, Ospedale Pediatrico Bambino Gesu’, Italy

Received November 2, 2011; Accepted June 26, 2013; Published September 4, 2013

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Funding: This study was supported in part by the Deutsche Forschungsgemeinschaft (HA 2959/3-1 within the group 1036 to SEH), and by a research grant from the Ernst-und-Berta-Grimmke foundation (SEH). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. No additional external funding was received for this study.

Competing Interests: The authors have declared that no competing interests exist.
the predominant regulatory subunit and carries out the guanine nucleotide exchange [5]. Subunits α, β and δ of eIF2B form a regulatory subcomplex that binds to phosphorylated eIF2α and down-regulate the activity of eIF2Bε [6] while eIF2Bγ enhances the activity of the ε-subunit [7].

The activity of eIF2B is highly controlled in many different cell systems in response to a variety of cellular conditions: Stressful situations (e.g. viral infections) decrease its activity, whereas stimulation of cells or tissues by insulin, growth factors or mitogens leads to its activation [9,9]. eIF2B is constitutively active and all known regulatory mechanism have exclusively inhibitory effects [10]. It can be controlled directly through changes in its phosphorylation status, or indirectly via phosphorylation of eIF2, since eIF2 (αF) is a potent competitive inhibitor of eIF2B (see for review [7]). Glycogen synthase kinase 3β (GSK-3β) is thought to play the greatest role in the inhibition of the activity of eIF2Bε by phosphorylating on Serine 535 [10,11]. Previously, phosphorylation of the ε-subunit of eIF2B by GSK-3β was identified in vitro in cardiomyocytes as an important signalling step mediating the antihypertrophic actions of GSK-3β through inhibition of protein translation [3].

GSK-3β usually requires for interaction with its substrates like eIF2B a phosphorylated serine or threonine residue located four amino acids C-terminal to its phosphorylation site [11]. In this context, DYRK1A and 2 have been described to physically interact with eIF2Bε in vitro, but their physiological role especially in cardiac hypertrophy still remains unclear. Following priming activity by DYRK2, the phosphorylation rate of tau by GSK-3β was 6 fold increased [11]. Even greater effect on the phosphorylation rate of Glycogen Synthase by GSK-3β was reported [12]. Phosphorylation of eIF2Bε on serine 535 by GSK-3β was only seen, when it has been previously phosphorylated by DYRK2 [12].

The DYRK family of protein kinases (dual specificity tyrosine(Y)-phosphorylation regulated kinase) are pleiotropic regulators of cellular functions and are evolutionary highly conserved [13]. In mammalians seven isoforms are known (DYRK1A, DYRK1B, DYRK1C, DYRK2, DYRK3, DYRK4A, DYRK4B). The sequences highly equal themselves in their kinase domain and the DYRK homology box [11]. DYRK1A is ubiquitously expressed in mammalians, whereas the expression of all other isoforms follows a tissue specific manner. Little is known yet about potential regulation mechanisms of DYRK kinases. They activate themselves during protein synthesis by autophosphorylation of a tyrosine residue in their activating domain [11]. After this step they act as constitutively active serine/threonine kinases. The autophosphorylation site is the only phosphorylation site described so far. DYRK2 is localized in the cytoplasm, whereas DYRK1A is predominantly found in the nucleus [3,11]. According to this distribution both kinases mainly phosphorylate substrates in their specific compartment.

In our previous work we could demonstrate that eIF2Bε is a potent stimulator of hypertrophic growth in cultured cardiomyocytes by increasing protein synthesis rate. In order to evaluate eIF2Bε as a therapeutic target for the treatment of cardiac hypertrophy we further investigated the related signalling mechanisms. This study is the first to show that the interaction of GSK-3β and its priming kinase DYRK2 is a potent regulatory mechanism of the activity of eIF2B and cardiac hypertrophy in vivo.

Materials and Methods

Generation of transgenic mice

To investigate the physiological role of eIF2Bε for myocardial hypertrophic response in vivo, we generated two transgenic mouse lines with cardiac restricted overexpression of eIF2Bε (eIF2Bε-TG), or overexpression of a mutant eIF2Bε-S535A (eIF2Bε-S535A-TG) which cannot be inactivated by phosphorylation through GSK-3β. cDNA encoding eIF2Bε or eIF2Bε-S535A was cloned into a plasmid containing the heart-specific β-myosin heavy chain (MHC) promoter and human growth hormone (hGH) poly(A)′ signal. DNA injections were performed into C57BL/6 embryos. Genomic DNA was isolated from mouse tail biopsies and analyzed by PCR with primers specific for the hGH poly(A)′ signal: (forward: 5'-GTC TAT TCG GGA ACC AAG CTG GAG TG-3′, reverse: 5'-ACA GCC ATC TAC TGA GTG GAC CCA AC-3′). Cardiac-specific overexpression of eIF2Bε was confirmed by Western blotting. Generation of transgenic animals as well as animal handling and experiments were performed according to the institutional guidelines of the University of Heidelberg and were approved by the animal experiment review board of the government of the state of Baden-Württemberg, Germany (approval number: 35-9185.81/G-76/07). All animals analyzed were males at the age of 3–4 months unless stated otherwise. Controls were performed with male littermates.

Echocardiography

Mice were anesthetized by isoflurane inhalation (~2% isoflurane), positioned on a warming pad (Fine Science Tools GmbH, Heidelberg, Germany) and allowed to breathe spontaneously. Echocardiography was performed by using an HDI 5000 CV echocardiography machine (ATL Ultrasound, Philips, Bothell, WA, USA) equipped with a 10-MHz probe. M-mode measurements of left ventricular dimensions were averaged from at least three cycles, using the edge-to-leading edge convention adopted by the American Society of Echocardiography. Digital images were analysed by using a HDI Lab (ATL, Philips, Bothell, WA, USA). Measurements were performed using Scion Image for Windows (Scion Corp). The investigator who conducted the echocardiography was blinded for the treatment status.

Left ventricular pressure volume measurements

Invasive assessment of hemodynamics was performed under anesthesia with isoflurane inhalation (initially 5%, after intubation 2% isoflurane). Mice were placed on controlled heating pads and mechanically ventilated. The right internal carotid artery was exposed, and a microtip catheter transducer (SPR-839, Millar Instruments, Houston, Tex, USA) was inserted into the right carotid artery and advanced into the left ventricle (LV) under pressure control. After stabilization, the pressure signals were recorded continuously with a pressure-volume conductance system (MPVS-300, Millar Instruments, Houston, Texas, USA) coupled with a PowerLab converter (PowerLab 4/20, ADInstruments Pty Ltd., Colorado Springs, CO, USA), stored by using suitable software (LabChart, ADInstruments), and displayed on a personal computer. PVAN software (Millar Instruments) was used for subsequent analysis of pressure-volume loops. The raw conductance volumes were corrected for parallel conductance by the hypertonic saline dilution method. For absolute volume measurements, the catheter was calibrated with known volumes of heparin-treated rat blood.
Immunoblot analyses

Immunoblot analyses were done for protein lysates prepared from the LV of wild type and transgenic mice (3 months unless otherwise stated) as well as for protein lysates prepared from primary cultures of ventricular cardiac myocytes. Protein concentration was measured using BCA protein assay (Interchim). Equal amounts of protein were separated with SDS-PAGE and transferred to a nitrocellulose membrane (Millipore). The membrane was stained with haematoxylin/eosin and myocyte size was measured as cross sectional area using the ImageJ software (Immagenics, NIH, USA). Picrosiris red staining was performed to detect collagen deposition. Evidence of fibrosis was evaluated using the ImageJ software (ImageJ, NIH, USA) in light microscopy pictures. Collagen area fraction in percent was then calculated as collagen area to tissue area ratio. TUNEL staining (In Situ Cell Death Detection Kit, Roche) was used to quantify cardiomyocyte apoptosis rate in LV tissue slides. In cultured cardiomyocytes trypsin blue staining (Sigma-Aldrich, Cat.-Nr. T8154) was performed to detect necrosis and apoptosis rates. For measuring capillary distribution in LV tissue we used haematoxylin/eosin staining and related the number of capillaries to tissue area.

Immunofluorescence was used to assess cytoskeleton structure and to quantify cell size in cultured cardiomyocytes. Phalloidin was used to stain actin filaments. Nuclei were counterstained with Hoechst 33342.

Quantitative real-time PCR

Total RNA was isolated from mouse left ventricular tissue using the Trizol reagent (Invitrogen). CDNA was synthesized with Revert Aid First strand cDNA synthesis kit (Fermentas). Real-time polymerase chain reaction (PCR) was performed using the LightCycler® system (Roche Diagnostics, Mannheim, Germany) according to the manufacturer’s instructions. All real-time PCR sample reactions were performed in triplicate and normalized to HPRT mRNA expression. Primers and specific probes were designed using the Universal Probe Library from Roche Diagnostics. The following primers were used for: HPRT 5′-TACCATGTGTAACG-3′ and 5′-GGTAGCGCTTGGGCTG-3′, probe # = 22; for ANF 5′-GATCTGTGAATTGTTTTTACCGATGCA-3′ and 5′-GCCTATCTTCCTACCCTACCCTTGCA-3′, probe # = 25; for BNP 5′-GTGCAAGGGGACATAAAG-3′ and 5′-GTCAGTCGCTTGGGTCATGG-3′, probe # = 13. A standard curve was run with the dilution series of the amplified fragment allowing for mRNA copy number calculation.

Immunohistochemistry

Immunohistochemistry was used for primary antibodies. Bands were quantified by densitometry using the Image J program.

Co-Immunoprecipitation

Co-immunoprecipitation (Co-IP) was used to study protein-protein interactions by immunoprecipitating a specific target protein and determining what other proteins are co-precipitated. Protein lysates were prepared in Pierce IP lysis buffer (Thermo scientific). Tissue lysates were pre-cleared by adding G-Sepharose (Roche) followed by incubation with eIF2B monoclonal antibody (Santa Cruz) for 4 hours at 4°C. Immune complexes were pulled down by incubating with Protein G-Sepharose for 4 hours at 4°C followed by washing with ice-cold lyses buffer 4–5 times, to eliminate non-specific interactions. Protein G-Sepharose bound immune-complexes were then resuspended in Laemmli sample buffer, boiled for 5 min and analyzed by western blotting. In negative control, no primary antibody was added.

Chronic β adrenergic stimulation via Isoproterenol

Mice were anesthetized with isoflurane, and Alzet osmotic minipumps (model 2002, Durect Corp, Cupertino, CA) were implanted subcutaneously in the neck during a brief surgery. Mice were randomized to receive isoproterenol HCl (I6504, Sigma-Aldrich, St. Louis, MO) at a dose of 30 μg/g per day dissolved in acidic isotonic saline, or isovolumic acidic saline alone (vehicle). Isoproterenol treatment was kept for 48 h respectively 14 days, as indicated.

Mouse model of ascending aortic banding

Male C57BL/6 at the age of 10 weeks were used in this experiment. Mice were sedated preliminary with medetomidine in cardiac myocyte culture medium containing Dulbecco’s modified Eagle’s medium (DMEM)/F-12 supplemented with 5% ascorbic acid, 100 μM 5-bromo-20-deoxyuridine. We used isoproterenol HCl (I6504, Sigma-Aldrich, St. Louis, MO) at a dose of 10 μg/g per day dissolved in acidic isotonic saline, or isovolumic acidic saline alone (vehicle). Isoproterenol treatment was kept for 48 h respectively 14 days, as indicated.

Primary culture of ventricular cardiac myocytes

Primary cultures of ventricular cardiac myocytes were prepared from 1–3-day old BR-Wistar rats (Charles River Laboratories) and purified using a discontinuous Percoll gradient. Cells were cultured in cardiac myocyte culture medium containing Dulbecco’s modified Eagle’s medium (DMEM)/F-12 supplemented with 5% horse serum, 4 μg/mL transferrin, 0.7 ng/mL sodium selenite, 2 g/L bovine serum albumin (fraction V), 3 mM pyruvic acid, 15 mM HEPES, 100 μM ascorbic acid, 100 μ/mL ampicillin, 5 μg/mL linoleic acid and 100 μM 5-bromo-20-deoxyuridine. We obtained cultures in which more than 95% of cells were myocytes. Culture medium was changed to serum-free at 24 h. The investigation was carried out in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised
siRNA transfection

For at least 5 h myocytes were cultured in serum and antibiotic free medium. For transfection of 2x10^6 cardiac myocytes in a 6 cm dish 250 pmol siRNA was diluted in 650 µL serum- and antibiotic free OptiMEM (Invitrogen). In another tube 4.5 µL of Lipofectamin 2000 (Invitrogen) was diluted in 200 µL OptiMEM and incubated for 5 min. After complex formation the solution and OptiMEM were added to the cells. The cells were incubated for 72 h at 37°C. In all siRNA mediated knockdown experiments myocytes transfected with non-specific (scrambled) siRNA were used as controls. siRNAs used were DYRK2 5’-AGA-AUA-AUC-UUC-3’ and siRNAs used were DYRK2 5’-AGA-AUA-AUC-UUC-3’, non-specific control siRNA 5’-AGG-UAG-UGU-AAU-CGC-CUU-GTT-3’. siRNAs were synthesized by MWG (Ebersberg, Germany).

Adenoviral infection

DYRK2 adenovirus (ABM Goods, Cat. Nr. 052224A) was used as vector to express DYRK2 protein in cardiomyocytes cultured in serum- and antibiotic free medium. Amplification was previously performed following manufacturer’s protocol. After incubation for 72 h at 37°C cells were analysed using immunofluorescence or immunoblot analyses.

Statistical analysis

Data are reported as mean ± SEM. Differences between groups were tested for statistical significance using unpaired 2 tailed t-test or multiple analyses of variance, when applicable. Student t-test or multiple analyses of variance, when applicable. Data were considered significant. Error bars in figures indicate standard error if not indicated otherwise.

Results

Overexpression of eIF2β does not alter cardiac phenotype in vivo, but lack of phosphorylation by GSK-3β elicits left ventricular hypertrophy

Confirmation of the overexpressions at protein level is shown in Figure 1A. Compared with C57BL/6 mice, eIF2β-eTG and eIF2β-eS237A-TG had normal litter size and showed no increased morbidity or mortality. Cardiac phenotyping was initiated at the age of 3–4 months. By contrast to our previous findings in vitro in cultured cardiac myocytes, overexpression of eIF2βeiniin vivo did not lead to myocardial hypertrophy. In fact, eIF2β-eTG animals displayed a cardiac phenotype comparable to wild type animals. However, baseline characterisation of eIF2B-S237A-TG revealed a spontaneous myocardial hypertrophy. Morphological characteristics for left ventricular hypertrophy in eIF2B-S237A-TG mice were already apparent to the eye in echocardiographic examination (Figure 1E) or when examining gross pathology of the excised hearts (Figure 1B) and could be verified consistently in all analyses (Figure 1F). Biometric as well as functional data were tested for statistical significance using unpaired 2 tailed t-test or multiple analyses of variance, when applicable. ANOVA was considered significant. Error bars in figures indicate standard error if not indicated otherwise.

Cardiac overexpression of eIF2βc does not change the response of the heart to pathologic stress induced by chronic isoproterenol treatment

After 14 days of isoproterenol stimulation wild type mice developed myocardial hypertrophy associated with an increase of LV to tibia length ratio about 36% (8.1 ± 0.5 mm/mm vs. 6.1 ± 0.1 mg/mm, p<0.01) and an increase of cardiac myocyte size about 44% (13997 ± 562 RU vs. 9699 ± 385 RU, p<0.01) as compared to vehicle treated animals. Myocardial growth in eIF2β-TG occurred in a comparable extent (for LV/TL by 28%: 8.2 ± 0.5 mm/mm vs. 6.0 ± 0.2 mm/mm, p<0.01; for myocyte size by 41%: 13870 ± 612 RU vs. 9847 ± 912 RU, p<0.01; Figure 2A), indicating presence of active counter-regulation mechanisms for increased protein levels of eIF2βc even under pathogenic stimulation. In eIF2B-S237A-TG, increase in LV to tibia length ratio and myocyte size was less distinct (for LV/TL by 20%: 8.9 ± 0.3 mm/mm vs. 6.9 ± 0.3 mg/mm, p<0.01; for myocyte size by 14%: 15354 ± 589 RU vs. 13694 ± 598 RU, p=n.s.) resulting in by trend still elevated values as compared to wild type or eIF2β-TG. However, LV weight to tibia length did not differ significantly anymore between the groups after chronic ß adrenergic stimulation. For all biometric and functional data see Table 3.

DYRK2 levels correlate strictly with phosphorylation of eIF2βc by GSK-3β

In conclusion of our results, we expected GSK-3β to be the key regulator controlling the activity of eIF2βc overexpression, since a loss of its phosphorylation site in eIF2B-S237A-TG animals lead to spontaneous cardiac hypertrophy. However, protein analysis of GSK-3β in left ventricular tissue did not show any differential expression or changes in phosphorylation status between the groups (Figure 3A). Faced with this surprising result, we were searching for alternative regulating mechanisms.

Four protein kinases have been previously described in vitro to directly phosphorylate eIF2βc and thereby have potential regulating action: Caseinkinase 1 and 2 (CK1 and CK2), Dual-specificity tyrosine phosphorylated and regulated kinase 1A and 2 (DYRK1A and DYRK2) and Glycogen synthase kinase 3B (GSK-3β) [10,11].
DYRK2 a Regulator of Cardiomyocyte Hypertrophy

We observed in this study, that increased expression of eIF2Bc in eIF2Bc-TG animals was accompanied by increased levels of DYRK2 under baseline conditions (Figure 3A) as well as under chronic administration of isoproterenol (Figure 2B). All other known phosphorylating kinases of eIF2Bc didn’t show any differential expression between the groups (Figure S2B). As mentioned before it was previously shown in vitro that the activity of GSK-3β is known to be mediated by DYRK2, suggesting a priming mechanism that makes eIF2Bc more susceptible for phosphorylation by GSK-3β. To evaluate a possible impact of other pathways in controlling eIF2Bc overexpression we analyzed expression and phosphorylation levels of kinases known to regulate protein synthesis. The activator of protein translation mTOR and its downstream targets 4E-BP and P70S6 were not differentially expressed in transgenic mice (Supplementary Figure 4).

Consistently throughout our experiments we could prove a strict positive correlation of DYRK2 levels and phosphorylation of eIF2Bc by GSK3-β on serine 535.

Following hypertrophic stimulation of C57BL/6 wildtype mice, eIF2Bc was higher expressed after 48 h but less phosphorylated by GSK-3β, which was consistently accompanied by a clearly decreased DYRK2 expression (Figure 4A). After 4 weeks, in a chronic compensated hypertrophic state the expression of eIF2Bc and DYRK2 returned to baseline levels both after isoproterenol treatment and aortic banding (data not shown).

Analysis of protein expression of eIF2Bc under conditions of heart failure in a swine model of atrial fibrillation (AF) revealed an unchanged expression of eIF2Bc. However, p-eIF2Bc(S535) was reduced (Figure S3), supporting again a regulatory role for modulating activity of eIF2Bc in response to cardiac stresses.

Further, after siRNA mediated knockdown of DYRK2 phosphorylation of eIF2Bc was reduced (Figure 4B) whereas it was increased after adenoviral induced overexpression of DYRK2 in cultivated cardiomyocytes (Figure 5A).

Additionally, direct physical interaction of eIF2Bc and DYRK2 was proven by co-immunoprecipitation using left ventricular tissue lysates from eIF2Bc-TG and eIF2Bc-S535A-TG (Figure 3B). Protein expression of DYRK1A and eIF2α did not differ in all groups.

Table 1. Biometric and functional measurements at the age of 3–4 months.

| Morphological parameters | wild type | eIF2Bc-TG | eIF2Bc-S535A-TG |
|--------------------------|-----------|------------|-----------------|
| n = 23                   | n = 13    | n = 10     |
| Heart/tibia length ratio [mg/mm] | 8.0±0.2 | 7.9±0.3 | 10.0±0.5 (p<0.001) |
| LV/tibia length ratio [mg/mm] | 6.0±0.2 | 6.1±0.2 | 7.5±0.4 (p<0.001) |
| RV/tibia length ratio [mg/mm] | 1.3±0.1 | 1.2±0.1 | 1.8±0.1 (p<0.001) |
| Lung/tibia length ratio [mg/mm] | 8.2±0.4 | 8.1±0.4 | 7.4±0.2 (p=n.s.) |
| Liver/tibia length ratio [mg/mm] | 81.2±2.2 | 74.0±2.8 | 84.2±5.0 (p=n.s.) |

| Echocardiographic parameters | n = 16 | n = 7 | n = 10 |
|-----------------------------|-------|------|-------|
| Heart rate [min⁻¹] | 459±12 | 470±30 | 500±9 (p<0.05) |
| AWTD [mm] | 0.48±0.01 | 0.51±0.01 | 0.65±0.02 (p<0.001) |
| End-diastolic diameter [mm] | 4.7±0.1 | 4.8±0.1 | 4.6±0.1 (p=n.s.) |
| PWTD [mm] | 0.50±0.01 | 0.52±0.01 | 0.66±0.02 (p<0.001) |
| Ejection fraction [%] | 76±1 | 76±1 | 74±1 (p=n.s.) |
| Fractional shortening [%] | 38±1 | 38±1 | 36±1 (p=n.s.) |

| Hemodynamic parameters | n = 20 | n = 12 | n = 8 |
|------------------------|-------|------|------|
| LVESP [mmHg] | 83±2 | 89±2 | 84±4 (p=n.s.) |
| LVEDP [mmHg] | 4±0.5 | 5±0.5 | 5±0.3 (p=n.s.) |
| Ejection fraction [%] | 60±2 | 61±3 | 59±3 (p=n.s.) |
| Stroke volume [ul] | 21±1 | 22±2 | 25±2 (p=n.s.) |
| LV dP/dtmax [mmHg/s] | 9198±521 | 8604±738 | 9786±606 (p=n.s.) |
| LV dP/dtmin [mmHg/s] | -8722±430 | -8575±622 | -8884±442 (p=n.s.) |
| Tau wave [ms] | 5.1±0.3 | 5.8±1.7 | 4.7±0.2 (p=n.s.) |

AWTD: Diastolic anterior wall thickness; PWTD: Diastolic posterior wall thickness; LVESP: left ventricular end systolic pressure; LVEDP: left ventricular end diastolic pressure. All values of eIF2Bc-TG showed no significant difference to wild type. *p = eIF2Bc-S535A-TG vs. wild type.

doi:10.1371/journal.pone.0070848.g001

doi:10.1371/journal.pone.0070848.g001
DYRK2 overexpression leads to spontaneously reduced cardiomyocyte size and diminishes hypertrophic response

In order to prove our hypothesis, that DYRK2 acts as a negative regulator of cardiomyocyte hypertrophy by priming inhibitory activity of GSK-3β on eIF2B, we induced adeno-

Table 2. Morphological and echocardiographic parameters at the age of 20 months.

| Morphological parameters | wild type | eIF2Bc-TG | eIF2Bc-S535A-TG |
|--------------------------|-----------|-----------|-----------------|
| Heart/tibia length ratio [mg/mm] | 10.5±0.4 | 9.2±0.3 | 12.0±0.4 (p<0.01) |
| LV/tibia length ratio [mg/mm] | 7.8±0.4 | 6.6±0.3 | 8.8±0.3 (p<0.05) |
| RV/tibia length ratio [mg/mm] | 1.4±0.0 | 1.6±0.1 | 1.5±0.1 (p=n.s.) |
| Lung/tibia length ratio [mg/mm] | 11.0±0.4 | 11.6±0.4 | 13.1±2.1 (p=n.s.) |
| Liver/tibia length ratio [mg/mm] | 116.2±6.2 | 115.0±5.7 | 103.6±7.5 (p=n.s.) |

| Echocardiographic parameters | n = 11 | n = 5 | n = 8 |
|-----------------------------|-------|------|------|
| Heart rate [min⁻¹] | 481±11 | 487±18 | 473±10 (p=n.s.) |
| AWTD [mm] | 0.57±0.02 | 0.55±0.02 | 0.75±0.02 (p<0.001) |
| End-diastolic diameter [mm] | 5.2±0.1 | 4.7±0.1 | 5.1±0.2 (p=n.s.) |
| PWTD [mm] | 0.59±0.02 | 0.57±0.01 | 0.82±0.03 (p<0.001) |
| Ejection fraction [%] | 68±2 | 72±2 | 60±2 (p<0.001) |
| Fractional shortening [%] | 32±1 | 35±1 | 27±1 (p<0.001) |

All values of eIF2Bc-TG showed no significant difference to wild type. *p = eIF2Bc-S535A-TG vs. wild type. AWTD: Diastolic anterior wall thickness; PWTD: Diastolic posterior wall thickness.
doi:10.1371/journal.pone.0070848.t002

Figure 2. Cardiac overexpression of eIF2Bc does not change the response of the heart to pathologic stress induced by chronic isoproterenol treatment (30 µg/g per day for 14 days) (A). WT n = 13/14 for vehicle/ISO, eIF2Bc-TG n = 9/8 for vehicle/ISO and eIF2Bc-S535A-TG n = 8/8 for vehicle/ISO. B: Expression of DYRK2 is increased after chronic administration of isoproterenol in left ventricular tissue of eIF2Bc-TG. Sections are exemplary details of the same blot.
doi:10.1371/journal.pone.0070848.g002

Discussion

Control of protein translation machinery is a fundamental regulatory mechanism in development of myocardial hypertrophic response. In this study we show that in vivo the activity of the e-subunit of Eukaryotic initiation factor 2B (eIF2B) underlies in the heart a tight regulatory network by interaction of GSK-3β and its priming kinase DYRK2. However, the release of their inhibitory influence in eIF2Bc-S535A-TG mice induces myocardial hypertrophy by increasing protein synthesis. Therefore we identify the Dual specificity tyrosine-phosphorylation-regulated kinase 2 (DYRK2) to regulate the repressive activities of GSK-3β on eIF2B, which determine the response of the heart upon pathologic injury.

Phosphorylation of eIF2Bc on Serine 535 by GSK-3β is required for controlling its prohypertrophic activity in vivo

By mediating the guanine nucleotide exchange, eIF2B regulates the initiation of mRNA translation in all eukaryotic cells. We have previously shown that phosphorylation of eIF2Bc on serine 535 by GSK-3 β controls cardiomyocyte hypertrophy following β-adrenergic stimulation in cultured cardiomyocytes [3]. In line with these previous findings, disinhibition of eIF2Bc from the influence of GSK-3β in eIF2Bc-S535A-TG mutant animals...
overexpression of the wild type form of eIF2B showed a distinct hypertrophic phenotype, which aggravated with aging and was associated with a trend for exaggerated hypertrophic response upon pathological stimulation. However, it was very surprising to see that, in contrast to our previously reported findings in cultured cardiomyocytes, even a very robust cardiac overexpression of eIF2B did neither change left ventricular hypertrophy on the organ or cellular level nor any of the classical hypertrophic marker genes. Moreover and even more unexpectedly, cardiac overexpression of eIF2B did not change minutely the response of the heart to pathologic stress, suggesting that a so far unrecognized and potent counter mechanism to control activity of eIF2B was not altered in the eIF2Bc-TG animals suggesting that another signalling molecule is the crucial factor for this observation.

| Table 3. Morphological and echocardiographic parameters after administration of isoproterenol or isovolumic vehicle for 14 days. |
|---------------------------------------------------------------|
| **Morphological parameters** | **isoproterenol** | **vehicle** | **isoproterenol** | **vehicle** | **isoproterenol** | **vehicle** |
|-------------------------------|-----------------|-------------|-----------------|-------------|-----------------|-------------|
| Heat/tibia length ratio       | 11.3±0.4        | 8.2±0.1     | 11.5±0.6        | 8.0±0.3     | 11.9±0.3       | 9.5±0.6     |
| [mg/mm]                       | (p<0.001)       | (p=n.s.)    | (p<0.001)       | (p<0.001)  | (p<0.001)      | (p<0.001)  |
| LV/tibia length ratio         | 8.1±0.3         | 6.1±0.1     | 8.2±0.5         | 6.0±0.2     | 8.9±0.3        | 6.9±0.3     |
| [mg/mm]                       | (p<0.001)       | (p=n.s.)    | (p<0.01)        | (p<0.01)   | (p<0.001)      | (p<0.001)  |
| RV/tibia length ratio         | 2.1±0.2         | 1.4±0.1     | 2.3±0.3         | 1.6±0.1     | 1.8±0.1        | 1.4±0.1     |
| [mg/mm]                       | (p<0.001)       | (p=n.s.)    | (p<0.05)        | (p<0.05)   | (p<0.05)       | (p<0.05)   |
| Lung/tibia length ratio       | 10.0±0.3        | 9.3±0.3     | 9.4±0.2         | 9.3±0.5     | 11.8±1.1       | 8.5±0.5     |
| [mg/mm]                       | (p=n.s.)        | (p=n.s.)    | (p=n.s.)        | (p=n.s.)   | (p<0.001)      | (p<0.001)  |
| Liver/tibia length ratio      | 101.2±3.9       | 95.6±3.5    | 102.9±3.9       | 85.9±3.3    | 97.4±3.6       | 104.9±3.3   |
| [mg/mm]                       | (p=n.s.)        | (p=n.s.)    | (p<0.001)       | (p<0.001)  | (p=n.s.)       | (p=n.s.)    |

| Echocardiographic parameters  | **n=13** | **n=14** | **n=9** | **n=8** | **n=8** | **n=8** |
|-------------------------------|----------|----------|--------|--------|--------|--------|
| Heart rate [min⁻¹]            | 556±10   | 478±21   | 553±17 | 475±18 | 508±23 | 507±25 |
| AWTD [mm]                     | 0.53±0.01| 0.48±0.03| 0.56±0.02| 0.47±0.02| 0.57±0.02| 0.56±0.02|
| End-diastolic diameter [mm]   | 5.3±0.2  | 4.9±0.1  | 5.1±0.1 | 4.5±0.1 | 5.3±0.1 | 4.7±0.2 |
| PWTD [mm]                     | 0.57±0.01| 0.50±0.02| 0.59±0.01| 0.47±0.02| 0.58±0.03| 0.57±0.03|
| Ejection fraction [%]         | 75±2     | 72±2     | 77±1   | 75±3   | 72±2   | 71±4   |
| Fractional shortening [%]     | 35±1     | 35±1     | 39±1   | 38±3   | 35±1   | 34±3   |

AWTD: Diastolic anterior wall thickness; PWTD: Diastolic posterior wall thickness.

DYRK2 acts as a negative regulator of cardiomyocyte growth by coordinating the repressor function of GSK-3β on eIF2Bc.

In search for the missing link of our observation and given the limited number of in vitro known interaction partners of eIF2Bc, we recapitulated that there is evidence that GSK-3b is phosphorylated at serine 539 and to act as a priming kinase for GSK-3β [11]. In this context, the kinases DYRK1A and DYRK2 have been both described in vitro to limit the number of in vitro known interaction partners of eIF2B and DYRK2 acts as a negative regulator of cardiomyocyte growth by coordinating the repressor function of GSK-3β [14]. In this context, the kinases DYRK1A and DYRK2 have been both described in vitro to limit the number of in vitro known interaction partners of eIF2B and DYRK2 acts as a negative regulator of cardiomyocyte growth by coordinating the repressor function of GSK-3β [14].
decreased expression of p-eIF2Bε(S535), as to expect in line with less activity of DYRK2 acting as priming kinase for GSK-3β. Corresponding results can be reported following adenoviral induced overexpression of DYRK2 which was accompanied by elevated levels of p-eIF2Bε(S535). Physical interaction of eIF2Bε and DYRK2 was proven by co-immunoprecipitation using left ventricular tissue lysates from our transgenic animals. In conclusion, these results support a coordinated response pattern with interaction of GSK-3β and its priming kinase DYRK2 phosphorylating eIF2Bε. In order to prove functional relevance of these regulatory axes we evaluated the effects on cardiomyocyte growth. Following adenoviral overexpression of DYRK2 we observed significantly reduced cardiomyocyte size under baseline conditions. Moreover DYRK2 overexpression had antihypertrophic effect and leads to reduced cardiomyocyte growth as response to phenylephrine stimulation. To complete our understanding of involved signalling pathways in regulating eIF2Bε overexpression, we analysed several main regulators of protein

Figure 3. Expression of DYRK2 is elevated in left ventricular tissue in eIF2Bε-TG. GSK-3β, DYRK1A and eIF2α were not different between groups. In each case, sections are exemplary details of the same blot (A). B: Immunoblots of co-immunoprecipitation demonstrating physical interaction of eIF2Bε and DYRK2 in left ventricular tissue of eIF2Bε-TG and eIF2Bε-S535A-TG.

doi:10.1371/journal.pone.0070848.g003
synthesis (Figure S4). The result that none of them was differentially expressed emphasizes for the first time the predominant role of DYRK2 as negative regulator of cardiomyocyte growth.

DYRK2 may play a physiological role in mediating cardiomyocyte hypertrophy in vivo

We could demonstrate in C57BL/6 wild type mice that phosphorylation of eIF2B by GSK-3β and DYRK2 expression are upregulated under conditions of increased protein translational demand, such as acute hypertrophic response. In the state of chronic compensation both levels return to baseline. In conclusion with our in vitro findings, the correlation of DYRK2 and phospho-eIF2B with conditions of increased protein synthesis are indicative for a physiological role of this regulatory axis in cardiac hypertrophy. Further evaluation of this hypothesis is necessary but goes beyond the scope of this investigation.

Protein translation as a potential target for treating LVH

Hypertrophic growth of cardiomyocytes is accompanied by increases in both protein synthesis and proteolysis rates; however the more rapid rate of protein synthesis relative to protein breakdown results in a net increase in protein accumulation and myocyte mass [16]. Increases in cellular protein synthesis rates can be due to a general augmentation of transcription, to an increase in post-transcriptional rates (i.e. splicing) or to accelerated translation rates [17]. Most proteins of the “fetal gene program” (including atrial natriuretic peptide (ANF), B-type natriuretic peptide, α-actin, myosin heavy chain (MHC) and others) are known to be regulated at the level of transcription [17]. Regarding the global increases in protein synthesis observed during cardiac hypertrophy, it is most likely that synthesis rates are regulated at the level of mRNA translation, as the availability of mRNA is not generally limited for increases in overall cell protein synthesis during growth [16]. The mRNA translation process can be boosted at the level of translation efficiency (i.e. the efficiency of translation of existing ribosomes) and of translation capacity (i.e. 

Figure 4. Levels of phospho(S535)-eIF2Bε and DYRK2 expression show strict correlation, indicating a coordinated response pattern following pathologic injury. A: In acute myocardial response 48 h after hypertrophic stimulation by isoproterenol or aortic banding of C57BL/6 wild type mice elf2Bε levels were elevated whereas phospho(S535)-elf2Bε and DYRK2 levels were significantly decreased. B: siRNA mediated knockdown of DYRK2 lead to reduced phospho(S535)-elf2Bε levels in vitro. Exemplary immunoblots of three individual experiments. doi:10.1371/journal.pone.0070848.g004

Figure 5. Adenoviral induced overexpression of DYRK2 in cultivated cardiomyocytes reduces baseline cell surface area and diminishes hypertrophic response to phenylephrine stimulation. A: Overexpression of DYRK2 is accompanied by increased levels of phospho(S535)-elf2Bε, and GSK-3β. B: Representative cultivated cardiomyocytes with reduced cell size and density of cytoskeleton following DYRK2 overexpression under baseline conditions. C: Cardiomyocyte size was significantly reduced in DYRK2 overexpressing cells compared to negative control under baseline conditions. Further, DYRK2 overexpression could almost inhibit the hypertrophic response to phenylephrine stimulation (n = 117 ad-Glox, n = 125 ad-DYRK2, n = 51 ad-Glox+PE, n = 68 ad-DYRK2+PE, *** <0.0001). doi:10.1371/journal.pone.0070848.g005
the amount of ribosomes actively translating mRNAs [17]. During the first phase of hypertrophic growth, the limiting step for the rise is the increase of translation initiation [10] which emphasizes again the potential role of eIF2Bc/DYRK2-axis as an anti-hypertrophic target.

Deregulation of the translational machinery is an important fundament of numerous human diseases as diverse as tissue hypertrophy, cancer, neurodegeneration and inflammation. Anyhow, no therapeutic option has been derived from this insight so far. Unravelling the mechanisms and control of mRNA translation will ultimately open up new therapeutic approaches for LVH, potentially involving a temporally and spatially restricted modulation of the activities of eIF2Be and DYRK2 in the myocardium.

Supporting Information

Figure S1 Protein expression of Ubiquitin is increased in left ventricular tissue of eIF2Be-S535A-TG, whereas eIF2Be-S535A-TG shows a comparable Ubiquitin expression to wild types. (TIF)

Figure S2 eIF2Be-S535A-TG showed a pronounced loss of EF in echocardiography at the age of 20 months compared to 4 months. (n = 11 WT, n = 5 eIF2Be-TG, n = 8 eIF2Be-S535A-TG. * p<0.05). (TIF)

Figure S3 p-eIF2Be is reduced under condition of heart failure in a swine model of atrial fibrillation (AF). AF was induced by pacemaker stimulation. The model and details about pacemaker implantation and further experimental settings have been described previously [19]. (TIF)

Figure S4 In order to explore other potential regulatory mechanisms of eIF2Be overexpression we analysed pathways known to have impact on protein synthesis rate. Immunoblot analyses are shown above. Neither expression nor phosphorylation levels of these kinases differed between the groups. (TIF)

Author Contributions

Conceived and designed the experiments: CSW MMO JS HAK SEH. Performed the experiments: CSW MMO MH MRS PM SJB KHW JHR SEH. Contributed reagents/materials/analysis tools: CSW MMO JS HAK SEH. Wrote the paper: CSW MMO SEH.

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