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Lack of $G\alpha_{i2}$ leads to dilative cardiomyopathy and increased mortality in β_1-adrenoceptor overexpressing mice

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Aims Inhibitory G (G_i) proteins have been proposed to be cardioprotective. We investigated effects of $G\alpha_{i2}$ knockout on cardiac function and survival in a murine heart failure model of cardiac β_1-adrenoceptor overexpression.

Methods and results β_1-transgenic mice lacking $G\alpha_{i2}$ (β_1-tg/$G\alpha_{i2}^{-/-}$) were compared with wild-type mice and littermates either overexpressing cardiac β_1-adrenoceptors (β_1-tg) or lacking $G\alpha_{i2}$ ($G\alpha_{i2}^{-/-}$). At 300 days, mortality of mice only lacking $G\alpha_{i2}$ was already higher compared with wild-type or β_1-tg, but similar to β_1-tg/$G\alpha_{i2}^{-/-}$, mice. Beyond 300 days, mortality of β_1-tg/$G\alpha_{i2}^{-/-}$ mice was enhanced compared with all other genotypes (mean survival time: 363 $\pm$ 21 days). At 300 days of age, echocardiography revealed similar cardiac function of wild-type, β_1-tg, and $G\alpha_{i2}^{-/-}$ mice, but significant impairment for β_1-tg/$G\alpha_{i2}^{-/-}$ mice (e.g. ejection fraction 14 $\pm$ 2 vs. 40 $\pm$ 4% in wild-type mice). Significantly increased ventricle-to-body weight ratio (0.71 $\pm$ 0.06 vs. 0.48 $\pm$ 0.02% in wild-type mice), left ventricular size (length 0.82 $\pm$ 0.04 vs. 0.66 $\pm$ 0.03 cm in wild types), and atrial natriuretic peptide and brain natriuretic peptide expression (mRNA: 2819 and 495% of wild-type mice, respectively) indicated hypertrophy. $G\alpha_{i3}$ was significantly up-regulated in $G\alpha_{i2}$ knockout mice (protein compared with wild type: 340 $\pm$ 90% in $G\alpha_{i2}^{-/-}$ and 394 $\pm$ 80% in β_1-tg/$G\alpha_{i2}^{-/-}$, respectively).

Conclusions $G\alpha_{i2}$ deficiency combined with cardiac β_1-adrenoceptor overexpression strongly impaired survival and cardiac function. At 300 days of age, β_1-adrenoceptor overexpression alone had not induced cardiac hypertrophy or dysfunction while there was overt cardiomyopathy in mice additionally lacking $G\alpha_{i2}$. We propose an enhanced effect of increased β_1-adrenergic drive by the lack of protection via $G\alpha_{i2}$. $G\alpha_{i3}$ up-regulation was not sufficient to compensate for $G\alpha_{i2}$ deficiency, suggesting an isoform-specific or a concentration-dependent mechanism.

Keywords Adrenergic receptor • Inhibitory G protein • Cardiomyopathy • Heart failure • Cardioprotection

1. Introduction

Norepinephrine concentrations and sympathetic drive are increased in human heart failure.[1–3] Although this helps to maintain contractile force, blood pressure, and blood flow to vital organs, it becomes detrimental in the long run.[4,5] β_1- and β_2-adrenoceptors are the most abundant cardiac adrenoceptors with the expression of β_1-adrenoceptors exceeding that of β_2-adrenoceptors by four-fold in the normal (human) heart.[4] β_1-adrenoceptors exclusively couple with stimulatory G proteins (G_s), whereas β_2-adrenoceptors have been shown to directly interact with both G_s and inhibitory G (G_i) proteins.[6,7] In human heart failure, expression of sarcolemmal β_1-adrenoceptors and their coupling with G_s decreases.[8] On the other hand, an increase of 'promiscuous' β_2- relative to β_1-adrenoceptors and an increased expression of G_i (particularly the isoform G_{i2}) are observed.[8,9] This latter finding can be interpreted as an attempt to

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shield the heart against catecholamines since Rau *et al.*[10] have shown that a G_{i2} increase similar to that found in human heart failure is sufficient to significantly reduce adenylyl cyclase-mediated cAMP production. This and other findings support the hypothesis that signalling via G_i proteins is cardioprotective.[11–14] Of interest, overexpression of both β_1- and β_2-adrenoceptors has been shown to induce cardiac hypertrophy and heart failure in mice, respectively.[15,16] However, levels of overexpression necessary to cause heart failure seem to be higher for β_2-adrenoceptors that in contrast to β_1-adrenoceptors couple with both G_s and G_i.[15,16] Nonetheless, cardioprotective effects mediated by G_i proteins are still a matter of debate, particularly regarding the cardiac condition, e.g. normal vs. pathological. To address this issue, we investigated whether lack of the catalytic G_{i2} subunit ($G\alpha_{i2}$) affects cardiac function and survival in the murine heart failure model of cardiac β_1-adrenoceptor overexpression compared with wild-type mice and mice either lacking $G\alpha_{i2}$ or overexpressing β_1. Parts of this work have already been published as a conference abstract.[17]

2. Methods

2.1 Mouse models used

Animals were kept in individually ventilated cages with a 12/12 h dark/light cycle and food and water *ad libitum*. Mice ubiquitary lacking $G\alpha_{i2}$ ($G\alpha_{i2}^{-/-}$) have originally been generated on a 129Sv background, but subsequently been backcrossed to C57BL/6J.[13,18,19] Mice with a cardiac-specific overexpression of the human β_1-adrenoceptor (β_1-tg) have originally been generated on a FVB/N background.[15] For the current study, we backcrossed this line on a C57BL/6J background (>10 generations) to allow for mating with $G\alpha_{i2}$ knockout mice to generate β_1-transgenic animals deficient of $G\alpha_{i2}^{-/-}$ (β_1-tg/$G\alpha_{i2}^{-/-}$). As control animals we used age-matched wild-type littermates. Animals of both sexes were used for our study. For genotyping, tail-clips from 3-week-old mice were used. Genomic DNA was prepared and genotyping PCR for $G\alpha_{i2}$ and the β_1-receptor was performed as described previously.[13,20] Animals were killed by cervical dislocation. Animal breeding, maintenance, and experiments were approved by the responsible federal state authority (Landesamt fuer Natur-, Umwelt- und Verbraucherschutz Nordrhein-Westfalen; reference: 87-51.04.2010.A078). All animal experiments comply with the guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes.

2.2 Survival analysis

All genotypes were monitored for a minimum period of 2 years. Kaplan–Meier survival curves were used to determine mean survival time of each mouse line. Spontaneous death was defined as the event of interest, while killing an animal was a censored event. Mice used for breeding were excluded from our analysis. Numbers of mice included in our survival analysis were 200, 34, 37, and 192 for C57BL/6 (wild type), $G\alpha_{i2}^{-/-}$, β_1-tg/$G\alpha_{i2}^{-/-}$, and β_1-tg mice, respectively. Numbers of spontaneous deaths that occurred during an observation period were 9 (C57BL/6), 10 ($G\alpha_{i2}^{-/-}$), 21 (β_1-tg/$G\alpha_{i2}^{-/-}$), and 22 (β_1-tg).

2.3 Echocardiography

At an age of 302 ± 19 days, mice ($n = 5–7$ of every genotype) were examined by echocardiography under light inhalation anaesthesia with oxygen and 1.5% isoflurane through a nose cap. Chests were epilated and the animals were placed on a heating table to prevent hypothermia and cardiodepressive effects. For the experiments, a commercial echocardiography system (Philips iE33 ultrasonic system, 'Qlab Cardiac Analysis' Software; Philips Healthcare, Hamburg, Germany) equipped with a 15 MHz linear array transducer (L15-io7) allowing frame rates of 270 Hz was used. The transducer was moved along the parasternal long and short axis of the left ventricle, and loops of 3 s duration were recorded in one-dimensional (M-mode) and two-dimensional planes. To monitor the heart rate of the animals and thus anaesthesia during measurements, an ECG was derived. For reconstructive three-dimensional echocardiography, multiple short-axis slices were recorded every 500 μm using a millimetre screw-tripod.[21,22]

2.4 Ventricle-to-body weight ratio

Before killing a mouse, its body weight was measured. For determining ventricular weight, hearts were excised immediately after killing by cervical dislocation, atria were cut, and intraventricular blood removed. We analysed 11, 8, 7, and 14 hearts of C57BL/6 (wild-type), $G\alpha_{i2}^{-/-}$, β_1-tg/$G\alpha_{i2}^{-/-}$, and β_1-tg mice, respectively, including those from mice examined by echocardiography.

2.5 Quantitative real-time PCR

For quantitative real-time PCR (qPCR), we used ventricles that were stored at −80°C immediately after excision. qPCR analysis was performed to determine relative ventricular mRNA expression levels of the cardiomyopathy markers atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP), the G_i proteins $G\alpha_{i2}$ and $G\alpha_{i3}$, and the cardiac protein kinase A (PKA) targets ryanodine receptor 2 (RYR2), troponin I (TnI, TNNI3), and phospholamban (PLB). All steps of analysis were performed following the manufacturer's protocol by QIAGEN (Hilden, Germany). mRNA isolation was performed with the RNeasy® Fibrous Tissue Kit (QIAGEN). Quality and quantity of the purified mRNA were controlled using a NanoDrop 8000 Spectrophotometer (Thermo Scientific, Waltham, MA, USA). For reverse transcription, the QuantiTect® Reverse Transcription Kit was used (QIAGEN). qPCR was run in triple repeats with the QuantiTect SYBR® Green PCR Kit (QIAGEN). Specific primer pairs for $G\alpha_{i2}$, BNP, RYR2, TNNI3, and PLB were designed using Roche Assay Design Center: $G\alpha_{i2}$: 5′-AAG ACC TGT CCG GTG TCA T-3′ for sense and 5′-GGG ATG TAG TCA CTC TGT GC-3′ for antisense. BNP: 5′-GTC AGT CGT TTG GGC TGT AAC-3′ for sense and 5′-AGA CCC AGG CAG AGT CAG AA-3′ for antisense. RYR2: 5′-TTC ACA CCT GTT CCT GTG GA-3′ for sense and 5′-TTT CTC TTA TCC TTT CCA GGT GA-3′ for antisense. TNNI3: 5′-GAG CCA CAC GCC AAG AAA-3′ for sense and 5′-GCC CCT TCT CTC CAC GTC-3′ for antisense. PLB: 5′-CTG TGA CGA TCA CCG AAG C-3′ for sense and 5′-TGG TCA AGA GAA AGA TAA AAA GTT GA-3′ for antisense. Primer pairs for $G\alpha_{i3}$ and ANP were reported previously.[23–25] S29 served as a housekeeping gene. The qPCR was started with an initial step of incubation at 95°C for 15 min. Next, 45 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 25 s, and elongation at 72°C for 10 s were run with a transition rate of 20°C per second. Finally, a melting curve analysis was applied to check for product purity at 64°C for 1 min with a transition rate of 0.1°C per second.

2.6 Western blot analysis

Ventricles from three different animals per genotype were isolated, separately homogenized, and individually analysed. Tissue was homogenized in protein lysis buffer containing 21 mM Tris–HCl, pH 8.3, 0.67% SDS, 238 mM β-mercapoethanol, and 0.2 mM PMSF. Gα protein isoform separation was performed in gels containing 6 M urea. In total, 20 μg per lysate was loaded. To verify $G\alpha_{i3}$ antibody specificity, ventricle lysates isolated from $G\alpha_{i3}$-deficient mice were loaded.[26] The proteins were visualized by immunodetection using the following primary antibodies described elsewhere:[27–30] rabbit anti-$G\alpha_{i2}$ (1 : 8000) and rabbit anti-$G\alpha_{i3}$ (1 : 5000). Equal loading was verified with antibodies against rabbit anti-β-actin (1 : 10 000). The protein levels of $G\alpha_{i2}$ and $G\alpha_{i3}$ were quantified using the densitometric analysis software (Image Lab; Bio-Rad, Gräfelfing, Germany) and were normalized to the β-actin levels of the same samples. For each ventricle, immune blots were run in triplicates or quadruplicates. Homogenates of the four different genotypes were always loaded on the same gel and analysed in parallel.

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2.7 Radioligand-binding experiments

Hearts from 4- to 6-month-old mice of both sexes were isolated in ice-cold phosphate buffer saline, frozen in liquid nitrogen, and stored at −80°C until membrane preparation. Ventricular tissue was homogenized in a 0.32 M sucrose solution and centrifuged for 11 min at 300 × *g*. Supernatants were centrifuged for 41 min at 80 000 × *g* and pellets resuspended in aqua destillata. All preparation steps were carried out at 4°C. Finally, aliquots of 0.5 mL were frozen in liquid nitrogen and stored at −80°C. Protein content was determined according to Lowry and ranged from 5.4 to 7.7 mg/mL. For saturation-binding experiments, membranes were incubated in Tris–HCl buffer (Tris 50 mM, pH 7.4; EDTA 5 mM) of a final volume of 1.5 mL at 23°C containing 50–300 μg protein. Eight concentrations (0.025–1.5 nM) of [^{3}H]CGP 12177 (specific activity: 37.7 Ci/mmol; PerkinElmer, Rodgau, Germany) were used to quantify β-adrenoceptor expression. Non-specific binding was measured in the presence of 10 μM propranolol or atropine (Sigma-Aldrich, Steinheim, Germany). Incubation was stopped after 90 min by rapid filtration through polyethylenimine (0.1%)-pretreated glass microfiber sheets using a Brandel cell harvester. Filter-bound radioactivity was detected by liquid scintillation counting. Fraction of β_2--adrenoceptor binding of [^{3}H]CGP 12177 was estimated in the presence of 70 nM ICI 118,551 (Sigma-Aldrich). Quality of the used homogenates was similar, as confirmed by muscarinic receptor levels determined with six concentrations (0.5–1.5 nM) of [^{3}H]*N*-methylscopolamine (specific activity: 84.1 Ci/mmol; PerkinElmer). Binding curves were analysed by non-linear curve fitting using the software GraphPad Prism®. Data points were fitted by a one-site-specific binding model.

2.8 Statistical analysis

Data are given as mean ± S.E.M. Survival times were calculated by Kaplan–Meier estimation. Differences were determined by the log-rank test. Cardiac functional parameters obtained from echocardiography and ventricle-to-body weight ratios were compared by ANOVA followed by *post hoc* tests (Bonferroni). mRNA expression ratios using C_t values obtained by qPCR were compared using the REST-2009® software.[31] Distribution between sexes was compared by Fisher's exact test. *P*-values <0.05 were defined to indicate statistically significant differences.

3. Results

3.1 Gross phenotype

Genotypes were distributed as expectable according to the Mendelian law (not shown). Overall distribution between sexes was nearly equal (51% male and 49% female) and did not differ between genotypes (not shown). All mice behaved normally and no overt phenotype was observed. Deaths occurred suddenly and were only in single cases preceded by unspecific symptoms like reduced movement or seclusive behaviour. Gastrointestinal symptoms as described previously for mice lacking $G\alpha_{i2}$ were not seen in our study, presumably due to keeping the mice in individually ventilated cages.[32]

3.2 Survival analysis

Kaplan–Meyer estimation revealed a significantly enhanced mortality of β_1-transgenic mice deficient of $G\alpha_{i2}^{-/-}$ (β_1-tg/$G\alpha_{i2}^{-/-}$) compared with all other genotypes (*Figure 1*). Mean survival time was reduced to 363 ± 21 days (22 deaths out of 37 mice in total) compared with 669 ± 31 for wild-type mice (9/200), 489 ± 37 for $G\alpha_{i2}^{-/-}$ (10/34), and 561 ± 20 for β_1-tg mice (21/192). Though survival time of mice only lacking $G\alpha_{i2}$ was significantly reduced, too, this effect was significantly more dramatic in β_1-tg/$G\alpha_{i2}^{-/-}$ mice ($P = 0.02$ in a log-rank test compared with $G\alpha_{i2}^{-/-}$). Of note, survival curves of β_1-tg/$G\alpha_{i2}^{-/-}$ and mice only lacking $G\alpha_{i2}$ were indistinguishable up to an age of ∼300 days. Similar to recent findings by another group, survival curves of wild-type and β_1-tg mice started to differ around an age of 13 months,[33] though in our study the difference in mean survival times derived from Kaplan–Meier analysis did not reach statistical significance. Fifty per cent of deaths in the β_1-tg/$G\alpha_{i2}^{-/-}$ group already occurred up to an age of 300 days. Since survival curves thus indicated a rather early onset of detrimental effects, we defined 300 days as the target age for further experiments. Moreover, this allowed for a sufficient number of animals available for *in vivo* examination. Of note, the choice of an age of 300 days excluded a considerable number of β_1-tg/$G\alpha_{i2}^{-/-}$ (and to a lesser extent $G\alpha_{i2}^{-/-}$) animals from further analysis due to early death. This might have biased the obtained data, e.g. if animals were still living at Day 300 because they have been less affected than those that had already died.

3.3 Echocardiographic analysis of cardiac morphology and function

At a mean age of 300 days (range: 273–326), the ventricle-to-body weight ratio of β_1-tg/$G\alpha_{i2}^{-/-}$ mice was significantly increased (0.71 ± 0.06%, $n = 5$) compared with wild-type mice (0.48 ± 0.02%, $n = 11$), $G\alpha_{i2}^{-/-}$ (0.50 ± 0.01%, $n = 8$), and β_1-tg mice (0.44 ± 0.01%, $n = 14$), thus indicating cardiac hypertrophy in β_1-transgenic mice lacking $G\alpha_{i2}$ (*Figure 2*). This was confirmed in echocardiographic examinations of anaesthetized mice of the same age. Ventricles of β_1-tg/$G\alpha_{i2}^{-/-}$ mice appeared to be clearly enlarged (see Supplementary material online, *Figure S1*). End-systolic and end-diastolic ventricular volumes were similar when comparing wild-type, $G\alpha_{i2}^{-/-}$, and β_1-tg mice, but significantly

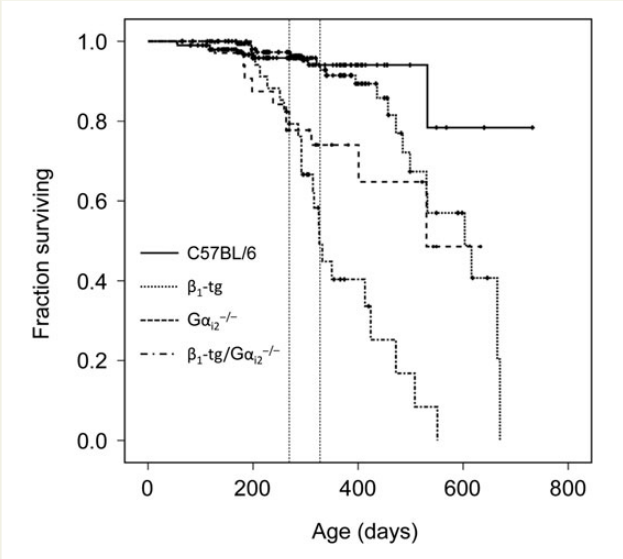

Figure 1 Kaplan–Meier survival curves of wild-type, knockout, and transgenic mice. Vertical bars indicate a censoring event (usually representing killing of an animal for experimental purposes). Mean survival times were significantly decreased for $G\alpha_{i2}^{-/-}$ and β_1-tg/$G\alpha_{i2}^{-/-}$ mice, each compared with all other genotypes. However, compared with $G\alpha_{i2}^{-/-}$ mice, survival time was significantly lower in β_1-tg/$G\alpha_{i2}^{-/-}$ mice (log-rank test). The number of animals underlying survival analysis was 200, 34, 37, and 192 for wild-type (C57BL/6), $G\alpha_{i2}^{-/-}$, β_1-tg/$G\alpha_{i2}^{-/-}$, and β_1-tg mice, respectively. Vertical dashed lines label age range of mice used for further investigations (*Figures 2, 3, 5–7*).

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enhanced in β_1-tg/$G\alpha_{i2}^{-/-}$ mice compared with all other genotypes (*Figure 3A* and *B*). Furthermore, ejection fraction was significantly reduced in β_1-tg/$G\alpha_{i2}^{-/-}$ mice (*Figure 3C*). Significantly increased ventricular length and reduced myocardial thickness in β_1-tg/$G\alpha_{i2}^{-/-}$ mice indicated a dilative cardiomyopathy (data not shown; compare Supplementary material online, *Figure S1*).

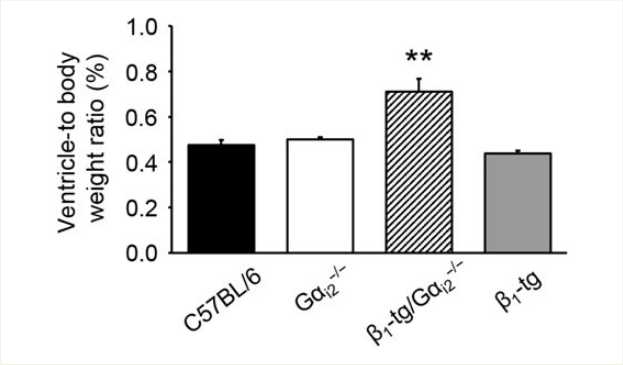

Figure 2 Ventricle-to-body weight ratios of knockout and transgenic mice indicate a significant cardiac hypertrophy of β_1-tg/$G\alpha_{i2}^{-/-}$ mice. The number of animals used for analysis was 11, 8, 5, and 14 for wild-type (C57BL/6), $G\alpha_{i2}^{-/-}$, β_1-tg/$G\alpha_{i2}^{-/-}$, and β_1-tg mice, respectively. Age range of examined mice was 301 ± 3 days. Asterisks indicate a significant difference compared with all other genotypes in Bonferroni post-tests following ANOVA ($P < 0.01$).

3.4 Expression of β-adrenoceptors and comparison to β-transgenic mice on an FVB/N background

Using saturation radioligand-binding experiments (*Figure 4*), we confirmed significant overexpression of β-adrenoceptors in 4- to 6-month-old β_1-tg mice (B_{max} 609 ± 18 vs. 8.4 ± 1.6 fmol/mg in C57BL/6 wild-type mice). In wild-type ventricles, ~80% of radioligand binding was due to β_1-adrenoceptors, while in transgenic mice virtually all detected receptors were β_1-adrenoceptors (data not shown). Of note, on the original FVB/N background, we found the overexpression level to be more than two-fold higher (B_{max}: 1425 ± 68 fmol/mg) than on a C57BL/6 background. Furthermore, in FVB/N-based β_1-transgenic mice, mean survival time was significantly reduced (402 ± 15 days). Similar to (C57BL/6-based) β_1-tg/$G\alpha_{i2}^{-/-}$ mice, ventricle-to-body weight ratio was increased (0.62 ± 0.03 vs. 0.52 ± 0.02% in FVB/N wild-type mice; $P < 0.05$; $n = 10$ and 7, respectively) and cardiac function impaired (e.g. ejection fraction: 21 ± 1 vs. 41 ± 2% in FVB/N wild-type mice; $P < 0.01$; $n = 5$ and 6, respectively) at a mean age of 307 ± 6 days (range 276–330).

3.5 Expression of G_i proteins

Expression of $G\alpha_{i2}$ mRNA was not detectable in $G\alpha_{i2}$ knockout mice (*Figure 5A*). We found that the lack of $G\alpha_{i2}$ was accompanied by an increased expression of $G\alpha_{i3}$ mRNA, both on a wild-type and on a β_1-transgenic background (*Figure 5B*). Expression of neither $G\alpha_{i2}$ nor $G\alpha_{i3}$ mRNA was significantly altered in hearts of mice being only transgenic for the β_1-adrenoceptor. Immunoblot analysis of ventricle

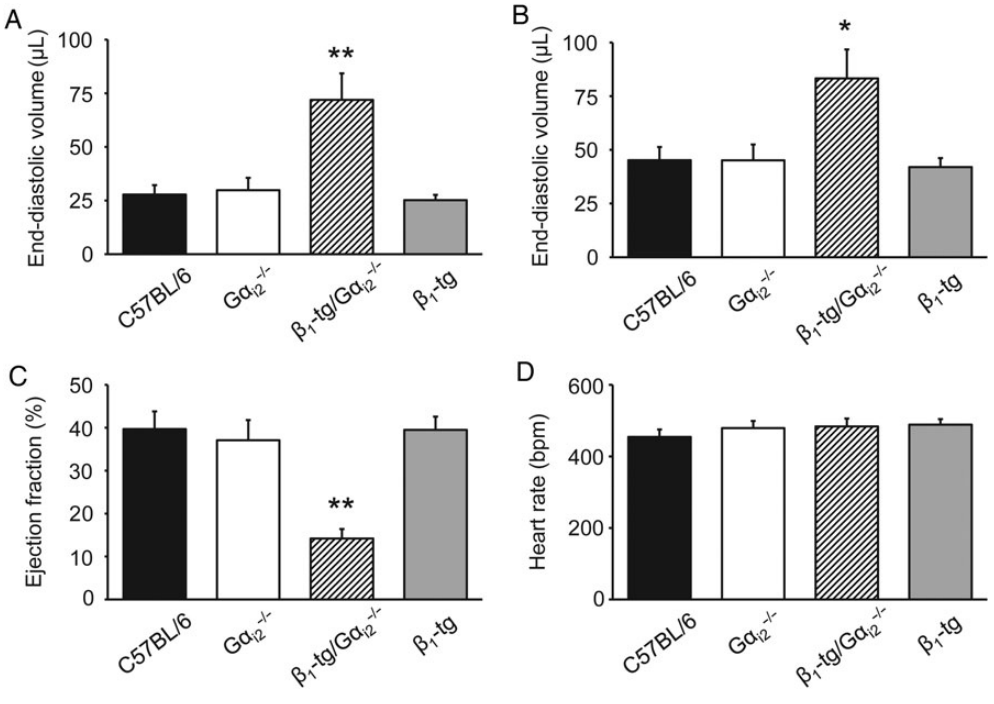

Figure 3 Morphological and functional parameters obtained by echocardiography. End-systolic (*A*) and -diastolic (*B*) left ventricular volumes and the ejection fraction (*C*) were significantly impaired in β_1-tg/$G\alpha_{i2}^{-/-}$ mice compared with all other genotypes indicating overt heart failure. Heart rate (*D*) was similar for all investigated genotypes. The number of animals used for analysis was 5, 6, 6, and 7 for wild-type (C57BL/6), $G\alpha_{i2}^{-/-}$, β_1-tg/$G\alpha_{i2}^{-/-}$, and β_1-tg mice, respectively. Age range of examined mice was 307 ± 3 days. Asterisks indicate significant differences compared with all other genotypes in Bonferroni post-tests following ANOVA (*$P < 0.05$, **$P < 0.01$).

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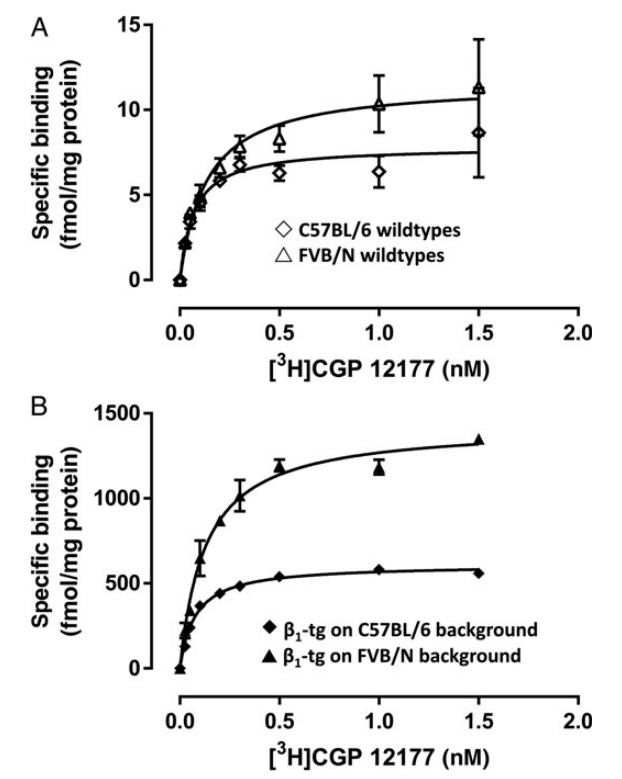

Figure 4 Ventricular β-adrenoceptor expression obtained by radioligand binding in (*A*) wild-type and (*B*) β_1 overexpressing mice (β_1-tg) at an age of 4–6 months. In both C57BL/6- and FVB/N-based β_1-tg mice, β-adrenoceptor expression was strikingly increased compared with the corresponding wild-type. Of note, the (over-) expression level was higher in tissue from transgenic mice with an FVB/N background. Each homogenate (10–12 ventricles per genotype) was used in three independent experiments in duplicates.

homogenates revealed $G\alpha_{i2}$ protein expression in ventricles of C57BL/6 and β_1-tg mice, whereas the $G\alpha_{i2}$ protein was absent in $G\alpha_{i2}^{-/-}$ and β_1-tg/$G\alpha_{i2}^{-/-}$ ventricles (*Figure 5C* and *E*). $G\alpha_{i3}$ protein levels were significantly up-regulated in $G\alpha_{i2}^{-/-}$ (340 ± 90%) and β_1-tg/$G\alpha_{i2}^{-/-}$ (394 ± 80%) ventricles compared with C57BL/6 and β_1-tg animals and absent in $G\alpha_{i3}$-deficient ventricles (*Figure 5D* and *F*).

3.6 Expression of hypertrophy markers and PKA targets

According to our morphological and functional findings, qPCR analysis revealed a significant up-regulation of the cardiomyopathy markers ANP (*Figure 6A*) and BNP (*Figure 6B*) in ventricular tissue of β_1-tg/$G\alpha_{i2}^{-/-}$ compared with wild-type mice. However, mRNA expression levels of ANP were also significantly increased in $G\alpha_{i2}^{-/-}$ mice and showed a tendency to be higher in β_1-tg animals (*Figure 6A*). BNP levels were unchanged in $G\alpha_{i2}^{-/-}$, but significantly increased in β_1-tg mice (*Figure 6B*). We tested for mRNA expression of the calcium release channel (ryanodine receptor) RYR2, the cardiac TnI TNNI3, and PLB as known targets of PKA-mediated phosphorylation (*Figure 7*). Compared with C57BL/6 wild-type mice, no differences were seen for RYR2 mRNA, but there was a significantly reduced expression of TnI in ventricles of β_1-tg/$G\alpha_{i2}^{-/-}$ (63 ± 7% of wild type), and of PLB in ventricles of both β_1-tg and β_1-tg/$G\alpha_{i2}^{-/-}$ (43 ± 6 and 47 ± 12% of wild type, respectively).

4. Discussion

In line with the idea of cardioprotective signalling mediated by G_i proteins, we show here that the lack of $G\alpha_{i2}$ in mice overexpressing cardiac β_1-adrenoceptors was detrimental: cardiac contractility was significantly depressed and survival time dramatically reduced. Mice deficient for either $G\alpha_{i2}$ or $G\alpha_{i3}$ have been reported to have no overt cardiac phenotype *in vivo* (echocardiography) and *ex vivo* (isolated whole hearts and myocytes).[34] This is in perfect agreement with our current data obtained from $G\alpha_{i2}$-deficient mice in the same range of age. Survival time of $G\alpha_{i2}$-deficient mice was reduced, but reduction was significantly more pronounced in β_1-tg/$G\alpha_{i2}^{-/-}$ mice. Of interest, survival curves of β_1-tg/$G\alpha_{i2}^{-/-}$ and mice only lacking $G\alpha_{i2}$ were indistinguishable up to an age of ~300 days. Since survival curves of β_1-tg mice start to drop rather late (this study and Lee *et al.*[33]), it is tempting to speculate that the early effect on mortality (up to Day 300) is due to $G\alpha_{i2}$ deficiency while the detrimental effect observed beyond 300 days of age is related to the cardiac overexpression of β_1-adrenoceptors. Given that cardiac function of mice only lacking $G\alpha_{i2}$ was unaffected at an age of 300 days, a cause of death other than reduced pump function has to be supposed. In a previous study, mice with both a $G\alpha_{i2}$ knockout and a cardiac-specific overexpression of β_2-adrenoceptors have been shown to be not viable.[13] This fatal outcome might be explained by direct coupling of β_2-adrenoceptors to G_i proteins. The drastic effect of $G\alpha_{i2}$ deficiency on top of cardiac β_1-adrenoceptor overexpression seen in our current study is rather surprising given that β_1-adrenoceptors are thought to exclusively couple with G_s proteins. However, recent work suggests a role for G_i in modulation of β_1-mediated effects. Melsom *et al.*[35] showed that inhibition of G_i proteins by pertussis toxin (PTX) not only enhanced cAMP accumulation following selective stimulation of either β_1- or β_2-adrenoceptors, but also increased inotropic potency of β_1- and β_2-adrenergic agonists, respectively. Another work by the same group indicates that G_i exerts intrinsic receptor-independent inhibitory activity on adenylyl cyclase.[36]

4.1 β_1-Adrenoceptor overexpression

Like in mice overexpressing the cardiac β_2-adrenoceptor, the phenotype of β_1-transgenic mice seems to depend on the extent of overexpression. A higher expression level of β_1-adrenoceptors led to an earlier and more pronounced hypertrophy of cardiomyocytes and a higher sensitivity of contractile response to dobutamine.[15] We found that after back-crossing on a C57BL/6 background, cardiac β-adrenoceptor overexpression was lower compared with FVB/N-based β_1-transgenic mice. This might explain why survival curves of β_1-transgenic mice on a C57BL/6 background start to drop later (in accordance to findings of another group that independently back-crossed the β_1-transgeni mice mouse of Engelhardt *et al.* on a C57BL/6 background).[15,33] In good agreement, cardiac function was already impaired at an age of 300 days in FVB/N-, but not in C57BL/6-based β_1-transgenic mice.

4.2 cAMP-dependent adrenergic signalling

That there was overt cardiomyopathy in (C57BL/6-based) β_1-transgenic mice additionally lacking $G\alpha_{i2}$ thus might be explained by a dual mechanism: increased β_1-adrenergic drive and lack of protection by $G\alpha_{i2}$. Engelhardt *et al.*[37] found an increased expression of the cardiac

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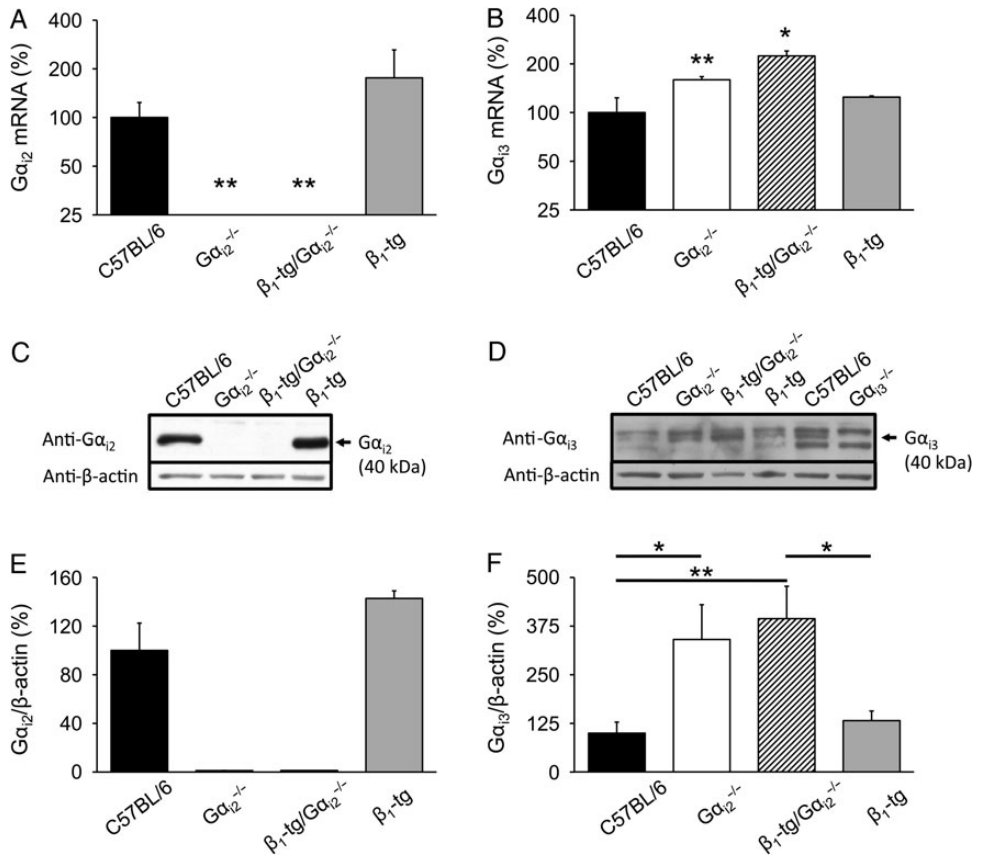

Figure 5 Ventricular expression of cardiac G_i. (*A*) $G\alpha_{i2}$ mRNA was not detectable in knockout mice on either wild-type or β_1-transgenic background and expressed at wild-type level in mice only transgenic for the β_1-adrenoceptor. (*B*) $G\alpha_{i3}$ mRNA expression was significantly increased compared with wild-type mice in animals lacking $G\alpha_{i2}$ on both a wild-type and β_1-transgenic background, respectively. The number of animals used for qPCR analysis was 3–4 for each genotype. Age range of examined mice was 302 $\pm$ 4 days. Asterisks indicate significant differences compared with mRNA expression levels of age-matched wild-type mice in an REST® analysis (*$P < 0.05$, **$P < 0.01$). (*C*) Representative immunoblots for the $G\alpha_{i2}$ protein in ventricle homogenates. The $G\alpha_{i2}$ protein was absent in $G\alpha_{i2}^{-/-}$ and β_1-tg/$G\alpha_{i2}^{-/-}$ mice. (*E*) Statistical analysis of $G\alpha_{i2}$ expression levels. (*D*) Representative immunoblot for the $G\alpha_{i3}$ protein in ventricle homogenates. To verify $G\alpha_{i3}$ antibody specificity, $G\alpha_{i3}$-deficient ventricle homogenates were loaded in addition. (*F*) Statistical analysis of $G\alpha_{i3}$ expression levels. A significant up-regulation of $G\alpha_{i3}$ protein levels was detectable in $G\alpha_{i2}^{-/-}$ and β_1-tg/$G\alpha_{i2}^{-/-}$ mice. For western blots, individual homogenates from three animals per genotype were analysed in 3–4 independent experiments. β-Actin was used to demonstrate equal loading of the gels. Asterisks indicate significant differences in Bonferroni post-tests following ANOVA (*$P < 0.05$; **$P < 0.01$).

Na^+-/H^+-exchanger NHE1 in β_1-adrenoceptor transgenic mice and showed that NHE1 inhibition prevented heart failure in this mouse model. Since NHE1 activity is modulated by cAMP, one might speculate that, in the β_1-overexpression model, lack of $G\alpha_{i2}$ further aggravates NHE1-dependent effects leading to accelerated development of cardiac dysfunction and eventually mortality in our study.[38] Though only an indirect hint, we looked at the expression of other potential phosphorylation targets at the mRNA level. Altered phosphorylation of the calcium release channel RYR2 has been attributed to heart failure and cardiac arrhythmia, though there is an ongoing controversial discussion.[39] In our mouse models, RYR2 mRNA expression was unchanged, but we cannot exclude that enhanced phosphorylation due to a maintained increase of β-adrenergic tone might be involved in the early mortality we observed in β_1-transgenic mice lacking $G\alpha_{i2}$. TnI seems to mediate both lusitropic and inotropic responses following β-adrenergic stimulation in murine hearts,[40] though the role of TnI and its phosphorylation in healthy and failing hearts is matter of debate.[41] We found a significant reduction of TnI expression at the mRNA level in β_1-transgenic mice lacking $G\alpha_{i2}$. This might be related to their detrimental outcome since it has been shown that TnI knockout leads to heart failure in adult mice.[42] Both β_1-transgenic mouse lines investigated in our study (β_1-tg and β_1-tg/$G\alpha_{i2}^{-/-}$) showed a significant reduction of PLB mRNA expression. Engelhardt *et al.*[43] found no alteration of PLB expression in β_1-transgenic mice at an age of 2 months, but an increased PLB phosphorylation indicating enhanced β-adrenergic signalling. The decrease of PLB expression observed at an age of ~9–10 months in our study might be compensatory by restoring the function of the SR calcium pump SERCA2A.[44] Indeed, knockout of PLB in β-transgenic mouse hearts has been shown to rescue its heart failure phenotype.[45] However, there seems to be a critical difference between mice and men because human PLB null has been shown to result in lethal cardiomyopathy.[46] Of note, PKA-dependent phosphorylation of β_2-adrenoceptors has been shown to drive a switch from G_s to G_i signalling,[47] though this mechanism is matter of

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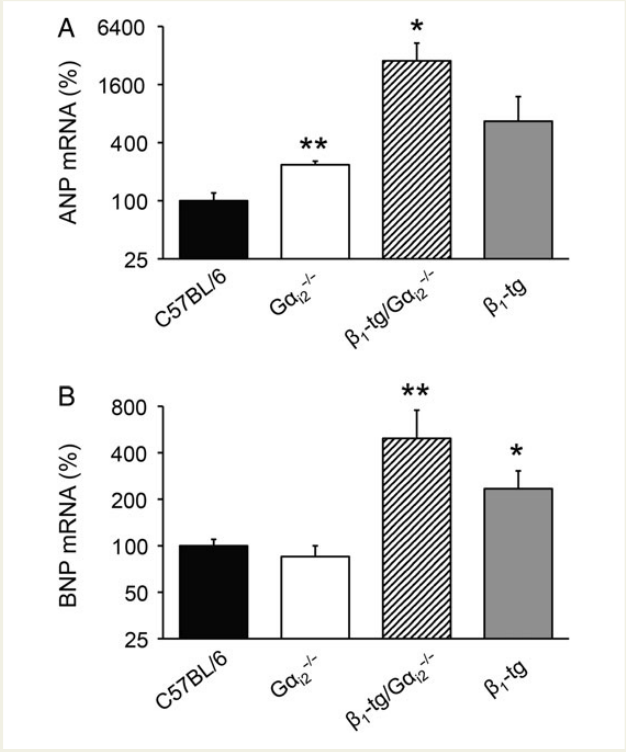

Figure 6 Expression of cardiac hypertrophy markers in murine ventricles. Increased mRNA expression of ANP (*A*) and BNP (*B*) compared with wild-type levels confirmed morphological parameters indicating cardiac hypertrophy in β_1-tg/$G\alpha_{i2}^{-/-}$ mice. The number of animals used for analysis was 3–4 for each genotype. Age range of examined mice was 302 ± 4 days. Asterisks indicate significant differences compared with mRNA expression levels of age-matched wild-type mice in an REST® analysis (*$P < 0.05$, **$P < 0.01$).

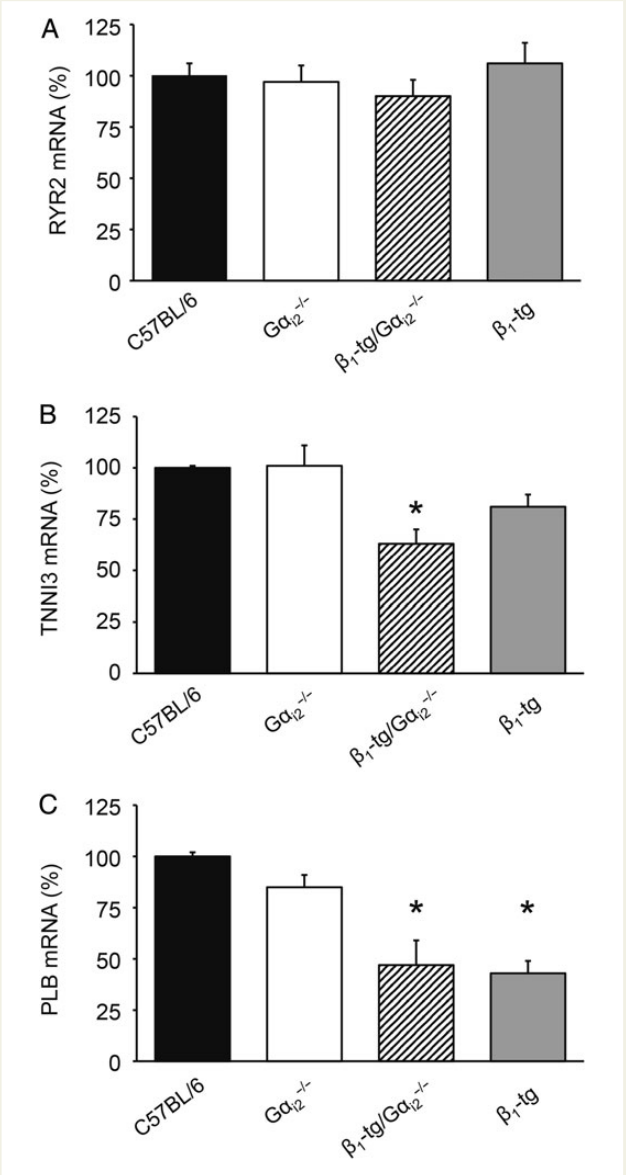

Figure 7 Ventricular mRNA expression of the PKA targets RYR2, TnI, and PLB. (*A*) RYR2 expression was similar in all genotypes. (*B*) TnI expression was significantly reduced in β_1-tg/$G\alpha_{i2}^{-/-}$ ventricles compared with wild-type (C57BL/6) tissue. (*C*) In both β_1-adrenoceptor overexpressing mouse lines (β_1-tg and β_1-tg/$G\alpha_{i2}^{-/-}$), ventricular PLB expression was significantly reduced compared with wild-type (C57BL/6) tissue. The number of animals used for analysis was 3–4 for each genotype. Age range of examined mice was 288 ± 9 days. Asterisks indicate significant differences compared with mRNA expression levels of age-matched wild-type mice in an REST® analysis (*$P < 0.05$).

debate. β_1-Adrenoceptor overexpression thus might enhance β_2-adrenoceptor-mediated G_i signalling. This would explain the pronounced effect of $G\alpha_{i2}$ deficiency in β_1-transgenic mice compared with mice with an otherwise wild-type background seen in our study.

4.3 G_i-mediated adrenergic signalling

Irrespective of the (disputed) role of β_2-adrenoceptor regulation by β_1-adrenoceptors, the accepted role of G_i proteins for β_2-adrenoceptor signalling should be considered. Communal *et al.*[48] have described opposing β-adrenoceptor-mediated effects on apoptosis in rat cardiomyocytes in terms of enhancement via β_1- but attenuation via β_2-adrenoceptors. Indeed, the combination of β_1-adrenoceptor blockers and β_2-adrenoceptor agonists has been shown to be superior to only β_1-adrenoceptor antagonists in treating cardiomyopathy in rodents.[49,50] Though the above-mentioned anti-apoptotic effect by β_2-adrenergic signalling was attributed to G_i-dependent activation of p38, the finding that overexpression of a dominant negative p38 isoform rescued cardiomyopathy of β_2- but not β_1-adrenoceptor transgenic mice argues against a role of this mechanism in our study.[51,52] Kohler *et al.*[14] have recently shown that following an ischaemia/reperfusion (I/R) protocol, cardiac infarct size was significantly increased in mice deficient for $G\alpha_{i2}$. Also in a murine I/R model, similar findings have been obtained with an induced cardiac expression of a G_i inhibitor peptide.[11] Taken together with our current study on β_1-adrenoceptor overexpressing mice, these data suggest that the cardioprotective role of G_i proteins might become evident under certain circumstances only, i.e. cardiac stress and/or disease. Not all studies support the idea of G_i proteins being protective in cardiomyopathy and heart failure. The beneficial effect of combined treatment with a β_1-adrenoceptor blocker and a β_2-adrenoceptor agonist following myocardial infarction in rats was also seen when using fenoterol that has been shown to mediate β_2-adrenergic effects independent of G_i proteins.[50,53] Of note, the

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reported G protein selectivity of stereoisomers of fenoterol has recently been challenged.[54] In a mouse model of dilative cardiomyopathy due to cre-recombinase overexpression, an increase of G_i signalling by knock-in of a mutated $G\alpha_{i2}$ did not improve but even worsen ventricular hypertrophy and mortality.[55] In a pathophysiologically more relevant model, Hussain *et al.*[56] found no change in G_i activity in rat heart failure following myocardial infarction: despite a significant increase of $G\alpha_{i2}$ protein by 50% G_i inhibition by PTX treatment, here did neither change baseline contractility nor inotropic response following β-adrenergic stimulation in ventricular strips from failing hearts.

4.4 Role of G_{i2} vs. G_{i3}

The putative differential roles of the most abundant cardiac G_i isoforms $G\alpha_{i2}$ and $G\alpha_{i3}$ are still a matter of debate. As mentioned above, mice with cardiac-specific overexpression of β_2-adrenoceptors were not viable, if they in addition were deficient of $G\alpha_{i2}$.[13] In the same study, heterozygous knockout of $G\alpha_{i2}$ in β_2-transgenic mice caused a reduced activity of ventricular L-type Ca^{2+} channels that was attributed to an increased expression of $G\alpha_{i3}$. These data led us to the hypothesis that $G\alpha_{i2}$ is cardioprotective, whereas $G\alpha_{i3}$ is rather of regulatory relevance. In a subsequent study, this hypothesis was supported by our finding that, in $G\alpha_{i2}$ knockout mice, cardiac $G\alpha_{i3}$ expression was up-regulated and accordingly ventricular Ca^{2+}-current density was decreased while, in mice lacking $G\alpha_{i3}$, Ca^{2+}-current density was enhanced despite an increased $G\alpha_{i2}$ expression.[24] Kohler *et al.*[14] confirmed an increased cardiac $G\alpha_{i2}$ expression in mice lacking $G\alpha_{i3}$ and found ventricular infarct size following ischaemia to be reduced here compared with wild-type mice or mice lacking $G\alpha_{i2}$.

5. Conclusion

In conclusion, our data support the idea of the G_i protein $G\alpha_{i2}$ to be cardioprotective. Of interest, this was observed in β_1-adrenoceptor overexpressing mice, i.e. a mouse model of cardiac stress not directly involving G_i signalling. The lack of $G\alpha_{i2}$ seemed to aggravate rather than cause cardiac dysfunction. The observed up-regulation of $G\alpha_{i3}$ was not sufficient to compensate for $G\alpha_{i2}$ deficiency, suggesting an isoform-specific or a concentration-dependent mechanism to be elucidated in further studies.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

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Conflict of interest: none declared.

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