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$G\alpha_{i2}$- and $G\alpha_{i3}$-Deficient Mice Display Opposite Severity of Myocardial Ischemia Reperfusion Injury

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Abstract

G-protein-coupled receptors (GPCRs) are the most abundant receptors in the heart and therefore are common targets for cardiovascular therapeutics. The activated GPCRs transduce their signals *via* heterotrimeric G-proteins. The four major families of G-proteins identified so far are specified through their α-subunit: $G\alpha_i$, $G\alpha_s$, $G\alpha_q$ and $G_{12/13}$. $G\alpha_i$-proteins have been reported to protect hearts from ischemia reperfusion injury. However, determining the individual impact of $G\alpha_{i2}$ or $G\alpha_{i3}$ on myocardial ischemia injury has not been clarified yet. Here, we first investigated expression of $G\alpha_{i2}$ and $G\alpha_{i3}$ on transcriptional level by quantitative PCR and on protein level by immunoblot analysis as well as by immunofluorescence in cardiac tissues of wild-type, $G\alpha_{i2}$-, and $G\alpha_{i3}$-deficient mice. $G\alpha_{i2}$ was expressed at higher levels than $G\alpha_{i3}$ in murine hearts, and irrespective of the isoform being knocked out we observed an up regulation of the remaining $G\alpha_i$-protein. Myocardial ischemia promptly regulated cardiac mRNA and with a slight delay protein levels of both $G\alpha_{i2}$ and $G\alpha_{i3}$, indicating important roles for both $G\alpha_i$ isoforms. Furthermore, ischemia reperfusion injury in $G\alpha_{i2}$- and $G\alpha_{i3}$-deficient mice exhibited opposite outcomes. Whereas the absence of $G\alpha_{i2}$ significantly increased the infarct size in the heart, the absence of $G\alpha_{i3}$ or the concomitant upregulation of $G\alpha_{i2}$ dramatically reduced cardiac infarction. In conclusion, we demonstrate for the first time that the genetic ablation of $G\alpha_i$ proteins has protective or deleterious effects on cardiac ischemia reperfusion injury depending on the isoform being absent.

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Introduction

Cardiovascular disease (CVD) in its various forms is a major cause of morbidity and mortality worldwide. Annually more than 17 million people die from CVD which represent approximately 29% of all deaths. Among those, 7.2 million die due to heart attack resulting from coronary heart disease (WHO 2012). Once considered a disease seen predominantly in industrial nations, nowadays myocardial infarction becomes more common also in developing countries [1]. This underlines the urgent need for strategies to protect the heart from ischemic injury.

In recent years a variety of cardio-protective drugs are used in clinical practice, such as β-adrenergic blockers or adenosine which all signal *via* G-protein-coupled receptors (GPCRs) [2]. Experimentally, compounds such as adenosine, opioids and bradykinin activating $G\alpha_i$-coupled receptors have been shown to attenuate myocardial reperfusion injury [3].

G_i-proteins belong to the family of heterotrimeric G-proteins consisting of α, β, and γ subunits of which Gα defines the nature of the G-protein. Upon ligand binding to the GPCR, the receptor catalyzes guanine nucleotide exchange in Gα which then leads to dissociation of Gβγ from the Gα subunit. It allows both entities to interact with downstream effectors, thereby initiating intracellular signaling necessary to elicit the biological response of the cell. Aside from G_i three other families of heterotrimeric G-proteins are known, namely G_s, G_q, and $G_{12/13}$. The G_i-family includes three closely-related Gα members, $G\alpha_{i1\text{-}3}$, each encoded by a single gene. The $G\alpha_{i1\text{-}3}$-isoforms share 85–95% of amino acid sequence identity and are characterized by their sensitivity towards pertussis toxin (PTX) [4,5]. $G\alpha_{i1}$, $G\alpha_{i2}$, and $G\alpha_{i3}$ display overlapping expression patterns with $G\alpha_{i2}$ and $G\alpha_{i3}$ abundantly expressed in the cardiovascular system [6,7]. Current research assumes that $G\alpha_{i2}$ and the quantitatively minor $G\alpha_{i3}$ isoform exhibit redundant physiological roles which may explain that single $G\alpha_{i2}$-deficient mice show only a relatively mild, and single $G\alpha_{i3}$-deficient mice no visible phenotype [8–10]. In line with the hypothesis that *in vivo* deletion of a single $G\alpha_i$-isoform can functionally be at least partially compensated by remaining $G\alpha_i$-isoforms, $G\alpha_{i2}/G\alpha_{i3}$-

double-deficient mice die *in utero* at early embryonic stages [11]. However, recent studies in mice lacking $G\alpha_{i2}$ or $G\alpha_{i3}$ disclose distinct biological key roles of these two $G\alpha_i$-isoforms [12]. In particular, defects of autophagic liver proteolysis, development of axial skeleton, and planar cell polarity in cochlear hair cells are solely caused by $G\alpha_{i3}$-deficiency [11,13,14]. Contrariwise, defects in skeletal muscle growth, thrombus formation and of various immune functions of leukocytes are detectable only in $G\alpha_{i2}$-deficient mice [15–18].

$G\alpha_{i2}$ has been suggested to play a significant role in ischemia reperfusion injury of the heart while a possible involvement of $G\alpha_{i3}$ has been neglected so far [19,20]. This study was undertaken to analyze isoform-specific consequences of $G\alpha_i$-deficiency on cardiac ischemic reperfusion injury in mice. Employing a well established and characterized murine *in vivo* model of heart ischemia and reperfusion in $G\alpha_i$-deficient mice [21] we show that $G\alpha_{i2}$-deficiency leads to massive myocardial ischemia reperfusion injury whereas $G\alpha_{i3}$-deficiency is highly protective in this scenario.

Materials and Methods

Ethics statement

Animal experiments were conducted in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals (FELASA). The protocol was approved by the committee on the Ethics of Animal Experiments of local authority "Regierungspräsidium Tübingen" (permit number: TO4/10). All surgery was performed under anaesthesia as described in the respective method parts and all efforts were made to minimize suffering.

$G\alpha_i$-deficient mouse strains

The generation and basal phenotypic characterization of $G\alpha_{i2}$-deficient and $G\alpha_{i3}$-deficient mice as well as their backcrossing on a C57BL/6 background were described elsewhere [10,11,16,17]. As controls wild-type C57BL/6 mice (WT) or littermates were used as indicated. $G\alpha_{i2}$-deficient mice were maintained in individually ventilated cages (IVCs) and $G\alpha_{i3}$-deficient mice under specific pathogen-free conditions (SPF) according to national guidelines for animal care at the animal facility of the University of Tübingen. The mice used were of either sex as we see no differences. All mice were between 8 to 12 weeks old except animals for expression analysis which were up to 14 weeks of age.

Murine model of myocardial ischemia

Murine model of myocardial ischemic reperfusion injury was performed as described previously [21]. Briefly, after receiving anesthesia (Pentobarbital, 80 mg/kg, i.p.), mice were placed on a temperature-controlled heating table. All animals were intubated, ventilated, and left parasternal thoracotomy was performed to lay open the left coronary artery. Deep anesthesia was controlled regularly to avoid suffering of mice. Coronary artery occlusion was achieved by using the previously described hanging weight system. After reperfusion a double staining technique using triphenyltetrazolium chloride (TTC) to mark vital and necrotic tissue and Evans Blue staining to negatively mark the AAR was used [22]. The extent of infarct sizes were determined by calculating the percentage of infarction compared to the area at risk (AAR) from 4–5 discs per heart [21]. Each group of animal consists of at least 6 mice. Planimetric determination of infarct size and AAR was performed using the ImageJ Software version 1.44p.

RT-PCR for transcriptional analysis

Tissue or whole blood cells were homogenized; RNA was isolated, and transcribed into cDNA. Transcriptional expression levels were measured using real-time reverse transcription polymerase chain reaction (iCycler CFX 96; Bio-Rad Laboratories, Munich, Germany) and normalized to two house-keeping genes, namely β-actin and GAPDH. To detect β-actin, GAPDH, $G\alpha_{i2}$ and $G\alpha_{i3}$ mRNA levels, following primers were used: GAPDH sense 5′-cga gaa tgg gaa gct tgt cat c-3′; GAPDH antisense 5′-cgg cct cac ccc att tg-3′; β-actin sense 5′-ctc tcc ctc acg cca tcc tg-3′; β-actin antisense 5′-tca cgc acg att tcc ctc tca g-3′; $G\alpha_{i2}$ sense 5′-gcc aac aag tac gac ggc a-3′; $G\alpha_{i2}$ antisense 5′-gta tct ctc acg ctt ctt gtg ct-3′; $G\alpha_{i3}$ sense 5′-atg aac cga atg cat gaa agc a-3′; $G\alpha_{i3}$ antisense 5′-ttt ggt gtc agt ggc aca ggt a-3′.

Immunoblot detection of $G\alpha_i$ proteins

After homogenization of tissue, samples were resuspended in RIPA buffer, and protein concentrations were measured by standard BCA method following the manufacturers' instructions (Thermoscientific, Illinois, USA). Protein amounts are indicated in the figure legends. Proteins were loaded on either urea-supplemented or 10% SDS polyacrylamide gels and blotted onto nitrocellulose membranes as described. The antibodies detecting $G\alpha_i$ proteins, i.e. anti-$G\alpha_{com}$, anti-$G\alpha_{i2}$, and anti-$G\alpha_{i3}$ were previously described [17,23]. Loading conditions were controlled by GAPDH. A horseradish peroxidase (HRP)-conjugated anti-rabbit IgG antibody served for immunodetection (Santa Cruz Biotechnology, Inc, USA, Santa Cruz). Immunoreactive bands were visualized by using an ECL detection system (GE Healthcare, Braunschweig, Germany). The levels of $G\alpha_{i2}$ and $G\alpha_{i3}$ in different organs from WTs; in heart tissue of WT, $G\alpha_{i2}^{-/-}$, and $G\alpha_{i3}^{-/-}$ mice; and in heart tissue at different time points at and after ischemic events were quantified by densitometric analysis using Image J 1.44p after normalizing to GAPDH level.

Immunofluorescence staining of myocardial tissue

Untreated hearts of WT, $G\alpha_{i2}^{-/-}$, and $G\alpha_{i3}^{-/-}$ mice and ischemic hearts (60 min) of mice after different reperfusion condition treatments (0, 60 and 120 min reperfusion) were excised and immediately frozen in Tissue-Tek® (Sakura Finetek, Netherland, Leiden). 0.5 µm thick cryostat sections were mounted on slides, fixed in 4% formaldehyde for 30 min, permeabilized with 0.1% Triton® X-100 (AppliChem, Germany, Darmstadt) for 10 min and blocked with 5% BSA in PBS for 45 min. For immunodetection of G-proteins in heart tissue, the previously described $G\alpha_i$ antibodies, i.e. anti-$G\alpha_{i2}$ and anti-$G\alpha_{i3}$, were used. DAPI (Invitrogen, USA, Oregon) was applied for nuclei detection. $G\alpha_{i2}$ and $G\alpha_{i3}$ signals were visualized with an Alexa 488-conjugated mouse anti-rabbit IgG (Invitrogen, USA, Oregon). The fluorescence imaging was performed with an Axiophot Zeiss microscope (Zeiss, Jena) using a digital camera with AxioVision 4.8 software.

Data analysis

Statistics were performed using one-way ANOVA with Bonferroni post test to determine group differences or unpaired student t test where appropriate. A value of $P<0.05$ was considered to be statistically significant.

Results

In order to get insights into the individual role of the two major $G\alpha_i$-proteins of the cardiovascular system, i.e. $G\alpha_{i2}$ and $G\alpha_{i3}$, in the development of myocardial ischemia reperfusion injury (MIRI)

we studied $G\alpha_{i2}^{-/-}$ and $G\alpha_{i3}^{-/-}$ mice in comparison to wild type controls in an acute murine model of heart ischemia and reperfusion [21].

Expression of $G\alpha_{i2}$ and $G\alpha_{i3}$ in murine organs

First, we compared expression levels of murine $G\alpha_{i2}$ and $G\alpha_{i3}$ in the heart and various other organs (Figure 1a). In the heart, both $G\alpha_i$-isoforms were detected on transcriptional and protein level (Figure 1a–c). Although low transcript levels were evident as compared to all other organs tested, significant protein expression was detectable in immunoblot analysis. Notably, as described for many organs and tissues [6,11,17] $G\alpha_{i2}$ is also the predominant isoform in the heart, although we found significant levels of $G\alpha_{i3}$ (Figure 1c).

Up regulation of $G\alpha_i$-proteins during myocardial IR

In accordance with its predominant expression, $G\alpha_{i2}$ has been reported to play an important role during ischemic injury [19]. Moreover, a recent study showed that enhancement of $G\alpha_{i2}$ signaling through loss of its negative regulation by RGS proteins protects the heart from ischemic injury [20]. Therefore, we wondered whether the different phases of myocardial ischemia reperfusion regulate expression levels of $G\alpha_{i2}$ since transcript levels were obtained 72 hrs after ischemia [19]. To address this question, the mice were exposed to a one hour period of ischemia followed by two hours of reperfusion (Figure 2a). At defined time points mice were sacrificed and their hearts analyzed. The cardiac tissue from the area at risk (AAR) was excised and transcript and protein expression levels of $G\alpha_{i2}$ (Figure 2b and c) and $G\alpha_{i3}$ (Figure 2d and e) were measured and compared to samples from sham-operated controls. The $G\alpha_{i2}$-specific mRNA was up regulated more than threefold after one hour of ischemia while there was no significant change in the protein level. In the following early phase of reperfusion $G\alpha_{i2}$ transcripts peaked with an eightfold increase with subsequent more than twofold increase in the protein level during late phase of reperfusion (Figure 2b and c). Similarly, $G\alpha_{i3}$ mRNA was also regulated during ischemia and reperfusion time in a similar manner but less intense (Figure 2d). Interestingly, protein levels increased statistically significant more than twofold during reperfusion phase (Figure 2e). To exclude that this enhanced expression is due to a massive influx of PMNs which highly express $G\alpha_{i2}$ and $G\alpha_{i3}$ the AAR sections were stained with anti-$G\alpha_i$- and subsequently with anti-CD15-antibodies (Figure S1). In fact, while leukocytes infiltrated into the heart tissue during the reperfusion phase a clearly enhanced $G\alpha_{i2}$- and $G\alpha_{i3}$-specific staining of cardiac tissue was evident. Taken together, the results may indicate that cardiac $G\alpha_{i2}$ and $G\alpha_{i3}$ play similar roles during ischemia reperfusion injury in the heart.

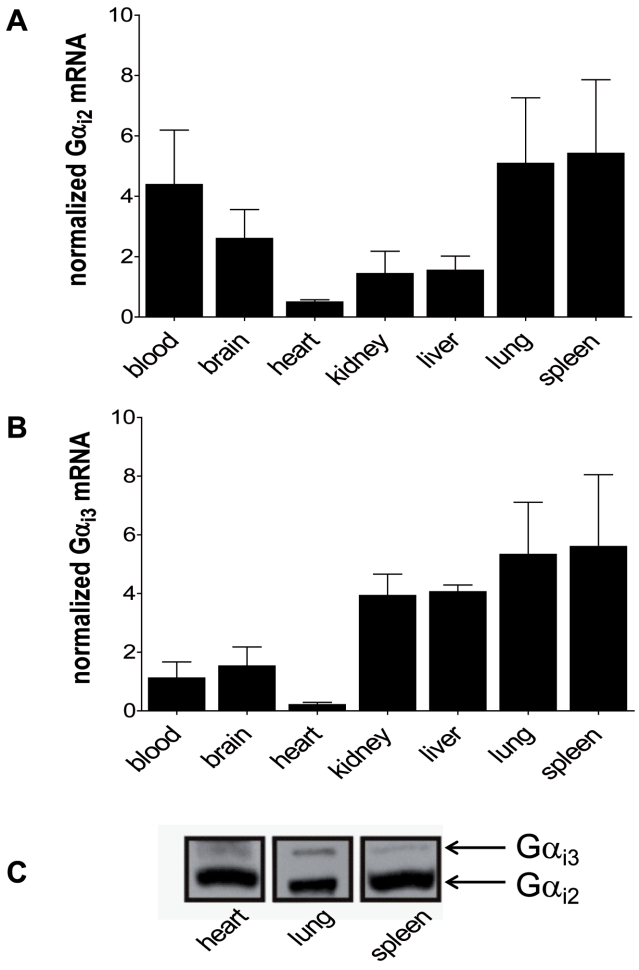

Figure 1. Expression of the two $G\alpha_i$-isoforms. RT-PCR analysis of various mouse organs. Transcriptional levels of $G\alpha_{i2}$ (**a**) and $G\alpha_{i3}$ (**b**) were normalized to β-actin and GAPDH. **c**. Immunoblot analysis of heart lysates from wildtype male mice with a $G\alpha_{common}$ antibody following urea supplemented SDS-PAGE and western blotting.
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$G\alpha_{i2}$-deficiency aggravates whereas $G\alpha_{i3}$-deficiency ameliorates IR-injury

To challenge the concept of redundancy of the two $G\alpha_i$ isoforms in IR-injury, we performed regional myocardial ischemia reperfusion in $G\alpha_{i2}$- (Figure 3) and $G\alpha_{i3}$-deficient mice (Figure 4). In this model the infarct size is determined by comparing the area of infarction within the area at risk (AAR) [21]. Vital and necrotic tissues within the AAR were identified by double staining with TTC and Evans Blue, respectively. The degree of myocardial destruction was calculated as percentage of infarcted myocardium to AAR. In this experimental setting WT mice show expected infarct sizes of 44.4±2.6%, whereas $G\alpha_{i2}^{-/-}$ mice displayed significantly increased infarct areas of 56.6±3.7% (Figure 3a). To illustrate the infarct size in a more visual fashion, one representative heart disc of each WT and $G\alpha_{i2}^{-/-}$ mice is depicted (Figure 3b). Concurrently, the area at risk was not significantly different between groups (Figure 3c). This supports and extends the previously described protective role of $G\alpha_{i2}$ in ischemia reperfusion [19,20].

Surprisingly, in $G\alpha_{i3}^{-/-}$ mice the extent of damage after myocardial IR-injury was dramatically decreased. $G\alpha_{i3}^{-/-}$ mice exhibited strong reduction in infarcted areas (25.0±3.5%) compared to controls (44.9±0.8%; Figure 4a) while there was no significant change in AAR (Figure 4c). Again, one representative heart disc of both groups is pictured (Figure 4b). These data reveal an up to now unknown protective mechanism in mice against IR injury in the absence of $G\alpha_{i3}$. Therefore, in contrast to current thinking, our data suggest that $G\alpha_{i2}$ and $G\alpha_{i3}$ play opposite instead of redundant roles in IR injury.

Increased expression of $G\alpha_{i2}$ in $G\alpha_{i3}$-deficient mice and vice versa

Previously, we detected an up regulation of the remaining isoform in different murine $G\alpha_i$-deficient tissues and cells for either $G\alpha_{i2}$ or $G\alpha_{i3}$ [11,17]. This is thought to represent an important mechanism contributing to functional redundancy and prompted

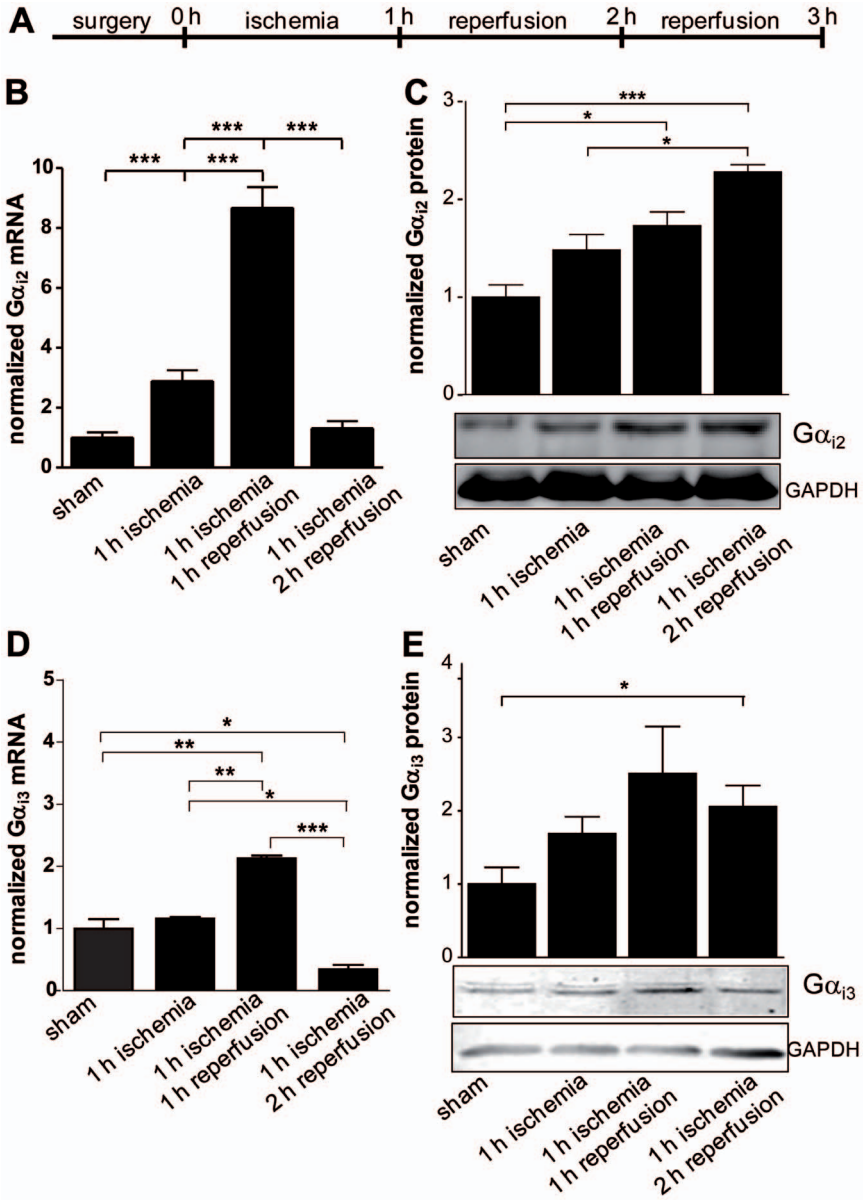

Figure 2. Increased expression of $G\alpha_{i2}$ and $G\alpha_{i3}$ during IR-injury. a. Schedule of the IR model with one hour ischemia followed by one or two hour reperfusion. **b**. Surgeries were performed as indicated with C57BL/6 male mice (WT). After the procedures AAR was excised and transcript levels of $G\alpha_{i2}$ were determined by quantitative PCR. **c**. Protein expression of $G\alpha_{i2}$ was analyzed by immunoblotting using a $G\alpha_{i2}$-specific antibody. Protein amounts were normalized to GAPDH. Protein amounts loaded were 60 µg. Shown are representative images. **d**. Transcript levels of $G\alpha_{i3}$ after surgeries as described in (a) were determined by quantitative PCR. **e**. Protein expression of $G\alpha_{i3}$ was analyzed by immunoblotting using $G\alpha_{i3}$-specific antibodies. Protein amounts were normalized to GAPDH. Protein amounts loaded 60 µg. Shown are representative images. Data are shown as mean ± SEM (n = 3); statistic was calculated with one-way ANOVA, with Bonferroni post test; $^*P \leq 0.05$; $^{**}P \leq 0.01$; $^{***}P \leq 0.001$ as indicated.
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us to ask whether the hearts of the knockout-mice lack compensatory up regulation of the remaining $G\alpha_i$-isoform. In a previous attempt we have analyzed hearts from knock out-mice using high resolution SDS-PAGE in combination with $G\alpha_{common}$ antibodies [6]. However; only partial resolution of $G\alpha_{i3}$ from $G\alpha_{i2}$ limited the value of the densitometric analysis. In particular, the upregulation of $G\alpha_{i3}$ in the absence of $G\alpha_{i2}$ might have been overestimated. To approach this question in a more rigorous way, we measured mRNA and protein levels of $G\alpha_{i2}$ in the cardiac tissue from $G\alpha_{i3}^{-/-}$ mice and *vice versa* (Figure 5 and 6). For assessment normalized transcript levels and immunoblot intensity of target proteins were compared to the housekeeping protein GAPDH. Isoform-specific antibodies were used for individual detection of $G\alpha_{i2}$ or $G\alpha_{i3}$ [11,17]. Both approaches showed significantly higher expression levels of $G\alpha_{i2}$ in $G\alpha_{i3}^{-/-}$ mice (Figure 5a and b) and *vice versa* $G\alpha_{i3}$ in $G\alpha_{i2}^{-/-}$ mice (Figure 6a and b). In $G\alpha_{i2}^{-/-}$ mice which lack the predominant $G\alpha_i$ isoform, $G\alpha_{i3}$ was up regulated more than twofold in cardiac tissue, and in $G\alpha_{i3}^{-/-}$ mice $G\alpha_{i2}$ expression levels were increased by 50%. To strengthen our finding on up regulated protein expression in either knockout mouse model, we performed immunofluorescence staining of murine heart tissue sections (Figure 5c and 6c). Validation of the primary and secondary antibodies is shown in Figure S2. In accordance with the RT-PCR and Western blot

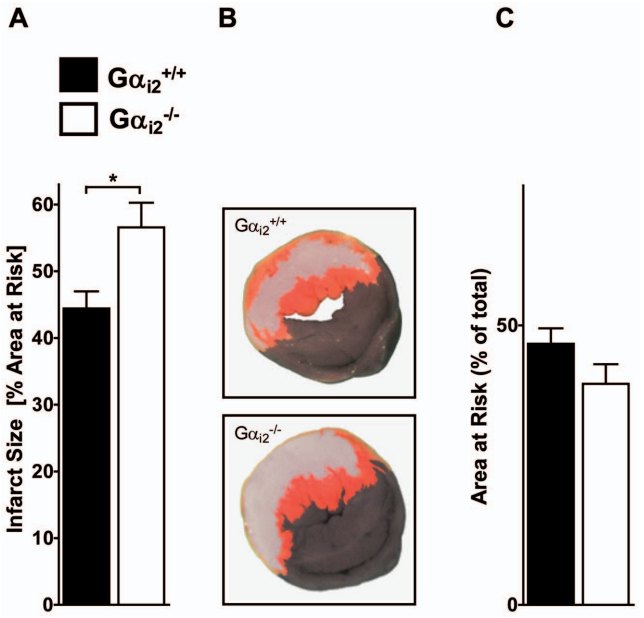

Figure 3. $G\alpha_{i2}$-deficiency aggravates IR-injury. a. $G\alpha_{i2}^{+/+}$ (n = 6) and $G\alpha_{i2}^{-/-}$ (n = 7) mice were exposed to one hour ischemia followed by two hours reperfusion. Hearts were stained with Evans Blue to determine the AAR and TTC to mark vital tissue (red) and necrotic tissue (white). Subsequently, infarct size was calculated as percentage of AAR (for details see Method's section). **b**. Representative heart slice of $G\alpha_{i2}^{+/+}$ and $G\alpha_{i2}^{-/-}$ mice are shown. These heart discs have an AAR of 50% ($G\alpha_{i2}^{+/+}$) and 50% ($G\alpha_{i2}^{-/-}$). The infarcted area was 41% ($G\alpha_{i2}^{+/+}$) and 58% ($G\alpha_{i2}^{-/-}$). **c**. Quantification of AAR as a percentage of the total heart disc (p = 0.14). Data in (a) and (c) are shown as mean ± SEM; statistic was calculated with t-test; *$P \leq 0.05$ as indicated.
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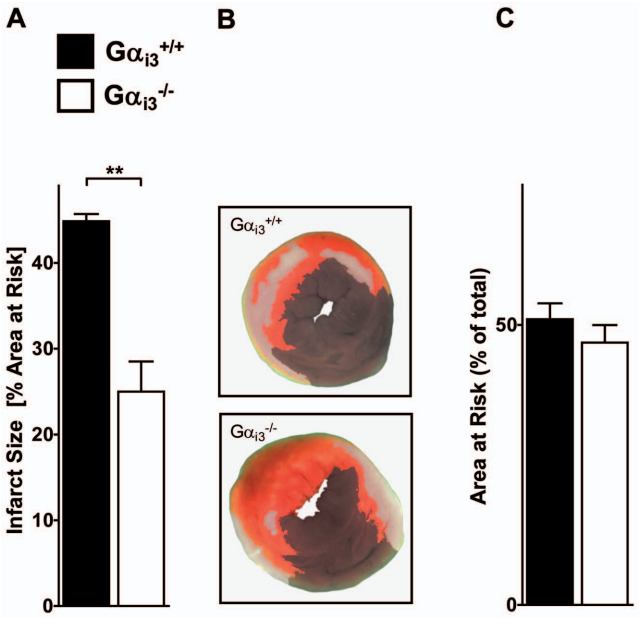

Figure 4. $G\alpha_{i3}$-deficiency ameliorates IR-injury. a. $G\alpha_{i3}^{+/+}$ (n = 7) and $G\alpha_{i3}^{-/-}$ (n = 6) mice were exposed to one hour ischemia followed by two hours reperfusion. Hearts were stained with Evans Blue to determine the AAR and TTC to mark vital tissue (red) and necrotic tissue (white). Subsequently, infarct size was calculated as percentage of AAR (for details see Method's section). **b**. Representative heart slice of $G\alpha_{i3}^{+/+}$ and $G\alpha_{i3}^{-/-}$ mice are shown. These heart discs have an AAR of 42% ($G\alpha_{i3}^{+/+}$) and 57% ($G\alpha_{i3}^{-/-}$). The infarcted area was 43% ($G\alpha_{i3}^{+/+}$) and 28% ($G\alpha_{i3}^{-/-}$). **c**. Quantification of AAR as a percentage of the total heart disc (p = 0.35). Data in (a) and (c) are shown as mean ± SEM; statistic was calculated with t-test; **$P \leq 0.01$ as indicated.
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results in either case signals of the remaining $G\alpha_i$-isoform were highly increased in heart tissue of the knock-out counterpart.

Therefore, we conclude that the compensatory up regulation of the remaining $G\alpha_i$ isoform did not mask the phenotype seen in the knock-out models.

Discussion

The role of G_i-protein-dependent receptor signaling in the cardiovascular system is still a matter of intense investigations. A variety of therapeutics acting on G_i-PCRs is currently in use for regulation of heart function and protection. Therefore, cardiovascular G_i-PCRs are the most targeted receptors in the pharmacological treatment of cardiac diseases [2,24]. In principle all these receptors can couple to two $G\alpha_i$-isoforms, i.e. $G\alpha_{i2}$ and $G\alpha_{i3}$. However, current thinking implies only $G\alpha_{i2}$ as the isoform responsible for eliciting biological effects whereas a role for $G\alpha_{i3}$ is neglected in this scenario. Hence, the aim of our study was to focus on a role for $G\alpha_{i3}$ in cardiac ischemia.

Here, we show for the first time that the absence of $G\alpha_{i2}$ or $G\alpha_{i3}$ have opposite effects on the severity of myocardial IR injury in knockout mice. In particular, $G\alpha_{i2}$-deficiency led to enhanced myocardial infarct size whereas the absence of $G\alpha_{i3}$ was highly protective. Whereas the first observation confirms and extends previous studies [19,20], the latter finding was unexpected. The increased infarct size visible in $G\alpha_{i2}$-deficient mice underlines a protective role of $G\alpha_{i2}$ signaling which was reported in previous studies making use of different experimental approaches. For instance, *in vivo* administration of PTX being considered a functional pan-G_i-inhibitor in combination with an infarct model demonstrated a cardio-protective effect of these G-proteins in rat hearts [25]. We performed similar experiments using our acute mouse model of 60 min. of regional myocardial ischemia followed by 120 min. reperfusion *in vivo* (Figure S3 and Methods S1). Interestingly, infarct sizes of the PTX-treated animals were even more pronounced as compared to those seen in $G\alpha_{i2}$-deficient mice, i.e. 67.0±4.8% *vs.* 56.6±3.7%, respectively, whereas the values for the controls in either group were almost the same (42.3±2.2% *vs.* 44.4±2.6%). The latter data argue for a reliable procedure as indicated by similar values in both control groups. PTX modifies $G\alpha_i$-proteins by ADP-ribosylation of a cystein residue in the extreme C-terminus of sensitive $G\alpha_i$-proteins. In the afore-mentioned study in rats [25] the degree of PTX-induced *in vivo* ADP-ribosylation of cardiac $G\alpha_i$-proteins was assessed by employing a radioactive *in vitro* approach. Interestingly, this analysis revealed that only a small subpopulation of G_i-proteins in the myocardial membrane was PTX-modified. This is a phenomenon we also see in our studies (data not shown). Since PTX modifies $G\alpha_i$-proteins with different efficiency, it cannot be excluded that PTX acted in a rather isoform selective way [26]. Moreover, different cells and tissues may exhibit variable sensitivity and kinetics towards PTX. Therefore it remains unclear which $G\alpha_i$-isoforms in which tissues and organs have contributed to the observed cardio-protective effect. Another study also targeted the interaction of GPCRs with cardiac G_i-proteins in a more specific approach [19]. Mice were created with a transgene expressing an inhibitory carboxyl-terminal 63 amino acid peptide of $G\alpha_{i2}$ in cardiac tissue acting in a dominant negative fashion. These mice, when subjected to ischemia/reperfusion induced heart injury, demonstrated an exacerbated ischemic injury as

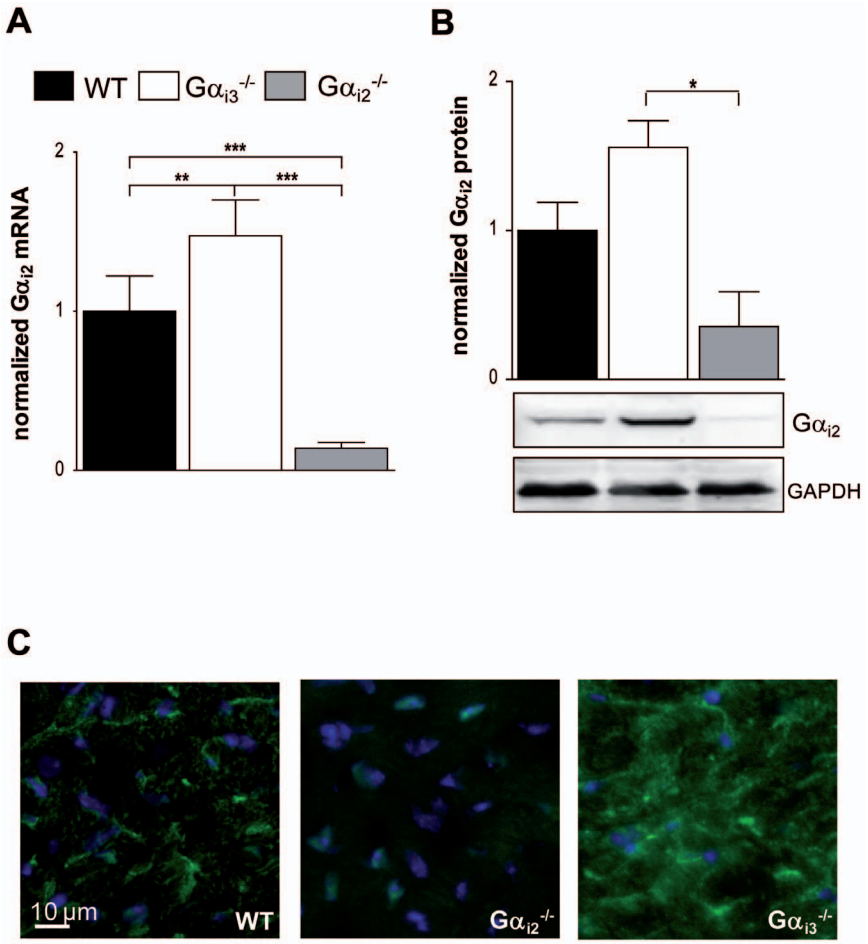

Figure 5. Expression of $G\alpha_{i2}$ in heart tissue of $G\alpha_{i3}$-deficient mice. a. mRNA levels of $G\alpha_{i2}$ in hearts of wildtype (WT), $G\alpha_{i2}$-deficient ($G\alpha_{i2}^{-/-}$) and $G\alpha_{i3}$-deficient male mice ($G\alpha_{i3}^{-/-}$) (n = 4). **b**. Representative immunoblots of heart from WT, $G\alpha_{i2}^{-/-}$ and $G\alpha_{i3}^{-/-}$ male mice detected with a $G\alpha_{i2}$-specific antibody. GAPDH was used to normalize the amount of protein. Protein amounts loaded 60 µg. The graph depicts the densitometric analysis (n = 3). **c**. Immunohistochemical staining of $G\alpha_{i2}$ in heart tissue of WT, $G\alpha_{i2}^{-/-}$ and $G\alpha_{i3}^{-/-}$ mice. Representative pictures are shown. Scale bar: 10 µm. Data are shown as mean ± SEM; statistic was calculated with one-way ANOVA, with Bonferroni post test; *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$ as indicated.
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compared to controls. Although the effects of the inhibitory $G\alpha_{i2}$-minigene on G_i-dependent signaling pathways were significant, the contribution of the $G\alpha_{i2}$- and $G\alpha_{i3}$-specific pathways to the observed cardio-protective effect was not investigated. In a recent paper a complementary genetic approach to study the effect of $G\alpha_{i2}$-signaling on cardiac ischemia *in vitro* was described [20]. Knock-in mice were examined in which the endogenous $G\alpha_{i2}$ gene was replaced with an RGS-insensitive G184S $G\alpha_{i2}$ mutant that was unable to interact with RGS proteins. This resulted in an enhancement of $G\alpha_{i2}$ signaling by reversal of its negative regulation by RGS proteins thereby protecting the heart from ischemic injury. Although this study was in accordance with the concept of $G\alpha_{i2}$-dependent protection of the heart, it ignored a possible role of $G\alpha_{i3}$. Moreover, these mice showed a dramatic and complex phenotype affecting the heart and several other organs which may produce secondary effects on heart function and resulting in premature death [27].

Similar concerns have been raised about the $G\alpha_{i2}$ knockout model that we have used in our current study. Initially, these mice have been reported to display a histopathological phenotype resembling ulcerative colitis and adenocarcinoma of the colon [10]. However, when these mice were housed under pathogen-free conditions no obvious signs of intestinal inflammation were visible during the course of the study and they did not show the previously reported lethality phenotype [17]. This allowed us to specifically study the roles of the two $G\alpha_i$-isoforms in cardiac ischemia injury *in vivo*.

Surprisingly, mice lacking $G\alpha_{i3}$ showed a significantly reduced infarct size following IR injury. It was intriguing that the deletion of one $G\alpha_i$ isoform results in the up regulation of the remaining ones. In fact, we detected an up regulation in heart tissue; a phenomenon we have observed previously in all tissues and cells we analyzed so far [6,11,17]. As a consequence, the particular knock-out model exhibits two important features, i.e. the deletion of the target $G\alpha_i$-isoform and the enhanced expression of the remaining ones. Therefore the deleterious or protective effect might not only be the result of the loss of one isoform but also the enhanced signaling of the remaining ones. For example, the increased infarct size seen in $G\alpha_{i2}$-deficient mice could either be due to missing $G\alpha_{i2}$ or over-expressed $G\alpha_{i3}$. Conversely, the reduced infarct size in $G\alpha_{i3}$-deficient mice could either be due to the over-expressed $G\alpha_{i2}$ or absent $G\alpha_{i3}$. In that respect, it will be interesting to re-evaluate the previous studies discussed above [19,20]. The main conclusions from these studies were to attach a predominant role of $G\alpha_{i2}$ in ischemia reperfusion injury. However, these studies ignored that an altered $G\alpha_{i2}$ signaling could affect

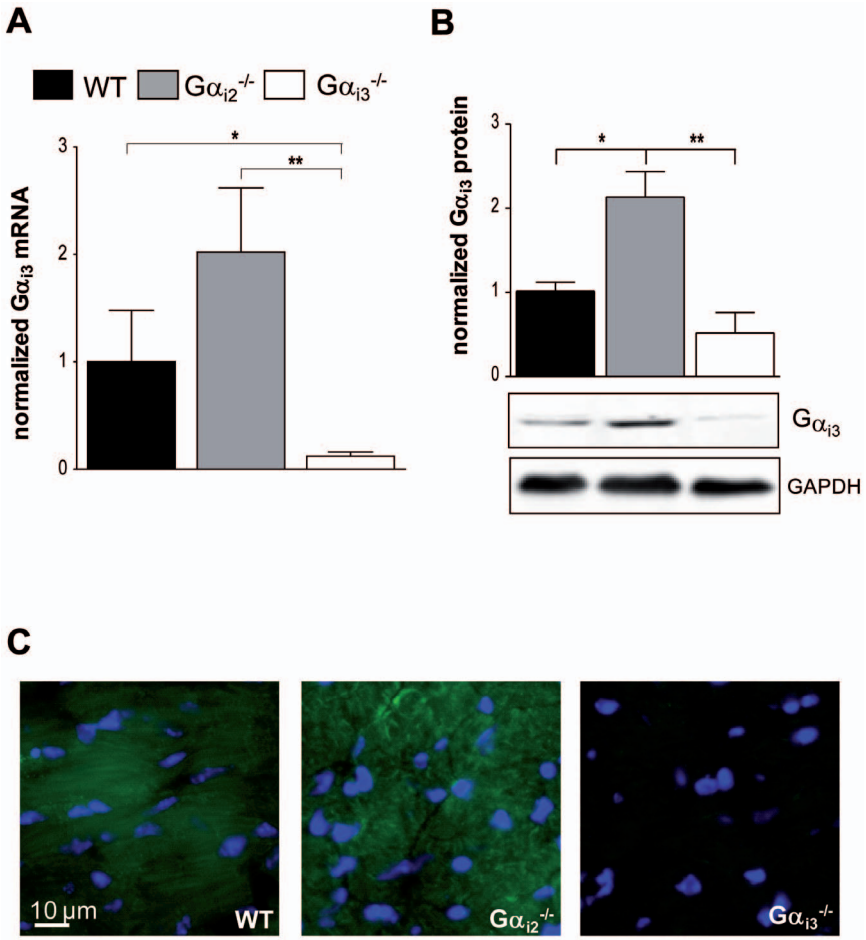

Figure 6. Expression of $G\alpha_{i3}$ in heart tissue of $G\alpha_{i2}$-deficient mice. a. mRNA levels of $G\alpha_{i3}$ in hearts of wildtype (WT), $G\alpha_{i2}$-deficient ($G\alpha_{i2}^{-/-}$) and $G\alpha_{i3}$-deficient male mice ($G\alpha_{i3}^{-/-}$) (n = 3). **b**. Representative immunoblots of heart from WT, $G\alpha_{i2}^{-/-}$ and $G\alpha_{i3}^{-/-}$ male mice detected with a $G\alpha_{i3}$-specific antibody. GAPDH was used to normalize the amount of protein. Protein amounts loaded 60 µg. The graph depicts the densitometric analysis (n = 3). **c**. Immunohistochemical staining of $G\alpha_{i3}$ in heart tissue of WT, $G\alpha_{i2}^{-/-}$ and $G\alpha_{i3}^{-/-}$ mice. Representative pictures are shown. Scale bar: 10 µm. Data are shown as mean ± SEM; statistic was calculated with one-way ANOVA, with Bonferroni post test; *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$ as indicated.
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$G\alpha_{i3}$ expression – as observed here and in previous studies – and signaling. This is of special importance since $G\alpha_{i3}$ has been shown to play crucial roles in both, its GDP-bound and GTP-bound form [28,29]. The current view is that $G\alpha_{i2}$ and $G\alpha_{i3}$ have largely overlapping roles. Some of our recent data contradict such a claim, showing that the absence of the minor $G\alpha_{i3}$ isoform cannot be compensated by the remaining $G\alpha_{i2}$ isoform [11,13,14]. G-protein signaling pathways come in at least two different shapes: a canonical and a non-canonical pathway which may mechanistically establish non-redundant distinct functions [29]. Future works have to concentrate on solving this question.

The current study displays intriguing and highly significant differences between the two $G\alpha_i$-isoforms albeit it employed a relatively small number of animals. One obvious limitation is the fact that global knockout animals, which lack the respective $G\alpha_i$-isoform in every tissue or organ, were studied. For future directions of research, in particular additional tools are required to decipher the specific functions of the two $G\alpha_i$ isoforms in cardiac and non-cardiac cells, e.g. cardiomyocytes, endothelial or immune cells. Ideally, experimental approaches may include detailed analyses of tissue-specific mouse models where the $G\alpha_i$ gene of interest is deleted in a constitutive or inducible manner. This allows elucidating the individual contribution of the $G\alpha_i$-isoforms to the ischemic reperfusion injury in the heart. Furthermore with this approach an up regulation of the remaining isoform may be prevented. Whereas an appropriate $G\alpha_{i2}$-model is available [13] the corresponding $G\alpha_{i3}$-mouse model has not been created so far.

In conclusion, we provide strong evidence that both the deficiency for $G\alpha_{i2}$ and for $G\alpha_{i3}$ has profound and opposite effects on IR injury in mice. This may open the rationale to develop biased G_iPCR drugs which may allow a different regulation of $G\alpha_{i2}$ and $G\alpha_{i3}$ by the same receptor.

Supporting Information

Figure S1 PMN infiltration in heart tissue during IR-injury. Surgeries in WT mice were performed as indicated. To stain infiltrated neutrophils, a source for the level changes in $G\alpha_i$ protein expression, immunohistochemistry with an anti-CD15 antibody was performed. Additionally, tissue was stained with **a**. $G\alpha_{i2}$- and **b**. $G\alpha_{i3}$-specific antibodies. Representative images are shown. Scale bar = 10 µm.
(TIF)

Figure S2 Control staining to test antibody specificity. To rule out unspecific binding of the used antibodies in heart tissue control staining were performed as follow. **a**. Staining of WT tissue with IgG antibody. **b**. Heart tissue from $G\alpha_{i2}^{-/-}$ mice was stained with anti-$G\alpha_{i2}$ antibody. **c**. Heart tissue from $G\alpha_{i3}^{-/-}$ mice was stained with anti-$G\alpha_{i3}$ antibody. Representative images are shown. Scale bar = 10 μm.
(TIF)

Figure S3 PTX treatment aggravates IR injury. **a**. WT mice were either injected i.p. with vehicle (n = 6) or Pertussis toxin (PTX)(see Methods S1) (n = 6) and 48 hours later exposed to one hour ischemia and one hour reperfusion. Hearts were counter-stained with Evans Blue to determine the AAR and TTC to mark vital tissue (red) and necrotic tissue (white). Subsequently, infarct size was calculated as percentage of AAR. **b**. Representative heart slice of WT mice treated with NaCl or PTX are shown. These heart discs have an infarcted area of 46% (WT+NaCl) and 69% (WT+PTX). Data in (a) are shown as mean ± SEM; statistic was calculated with t-test; ***$P \leq 0.001$ as indicated.
(TIF)

Methods S1 Pertussis Toxin treatment.
(DOCX)

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Author Contributions

Conceived and designed the experiments: DK VD CB LB PR SBH BN. Performed the experiments: DK VD CB TE AN JR TG. Analyzed the data: DK VD TG SBH. Contributed reagents/materials/analysis tools: LB PR BN. Wrote the paper: DK VD TE SBH BN.

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