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GRK2 blockade with β ARKct is essential for cardiac β_2 -adrenergic receptor signaling towards increased contractility

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Abstract

Background: β_1 - and β_2 -adrenergic receptors (ARs) play distinct roles in the heart, e.g. β_1 AR is pro-contractile and pro-apoptotic but β_2 AR anti-apoptotic and only weakly pro-contractile. G protein coupled receptor kinase (GRK)-2 desensitizes and opposes β AR pro-contractile signaling by phosphorylating the receptor and inducing beta-arrestin (β arr) binding. We posited herein that GRK2 blockade might enhance the pro-contractile signaling of the β_2 AR subtype in the heart. We tested the effects of cardiac-targeted GRK2 inhibition in vivo exclusively on β_2 AR signaling under normal conditions and in heart failure (HF).

Results: We crossed β_1 AR knockout (B1KO) mice with cardiac-specific transgenic mice expressing the β ARKct, a known GRK2 inhibitor, and studied the offspring under normal conditions and in post-myocardial infarction (MI). β ARKct expression in vivo proved essential for β_2 AR-dependent contractile function, as β_2 AR stimulation with isoproterenol fails to increase contractility in either healthy or post-MI B1KO mice and it only does so in the presence of β ARKct. The main underlying mechanism for this is blockade of the interaction of phosphodiesterase (PDE) type 4D with the cardiac β_2 AR, which is normally mediated by the actions of GRK2 and β arrs on the receptor. The molecular "brake" that PDE4D poses on β_2 AR signaling to contractility stimulation is thus "released". Regarding the other beneficial functions of cardiac β_2 AR, β ARKct increased overall survival of the post-MI B1KO mice progressing to HF, via a decrease in cardiac apoptosis and an increase in wound healing-associated inflammation early (at 24 hrs) post-MI. However, these effects disappear by 4 weeks post-MI, and, in their place, upregulation of the other major GRK in the heart, GRK5, is observed.

Conclusions: GRK2 inhibition in vivo with β ARKct is absolutely essential for cardiac β_2 AR pro-contractile signaling and function. In addition, β_2 AR anti-apoptotic signaling in post-MI HF is augmented by β ARKct, although this effect is short-lived.

Keywords: Cardiac β_2 -adrenergic receptor, Pro-contractile signaling, Post-myocardial infarction survival, GRK2 inhibition, GRK5

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Background

Despite recent advances in prevention and management of heart disease, death due to post-myocardial infarction (MI) heart failure (HF) continues to rise and new treatments are needed [1]. β_1 - and β_2 -adrenergic receptors (ARs) are the main stimulatory receptors of cardiac function but are now known to play clearly distinct roles in cardiac physiology and pathology [2-5]. For instance, cardiomyocyte contraction is readily stimulated by β_1 ARs but not β_2 ARs and β_1 AR signaling is generally considered pro-apoptotic whereas β_2 AR signaling anti-apoptotic in the heart [2-5]. These differences might be explained by differences in the signaling complexes assembled by activation of these two β ARs: β_1 AR forms a complex with phosphodiesterase (PDE) 4D8 directly when inactive, and agonist binding dissociates it [6,7]. Additionally, β_1 AR does not readily bind the receptor adapter proteins beta-arrestins (β arrs) following its agonist-promoted phosphorylation by G protein coupled receptor kinases (GRKs), the most prominent members of which in the heart are GRK2 and -5 [8-11]. In contrast, β_2 AR recruits another PDE variant upon its agonist activation, PDE4D5, via its interaction with β arrs following its GRK-dependent phosphorylation [6,7,12-16]. PDE recruitment to the receptor's signaling complex plays a crucial role in compartmentalizing the cyclic adenosine monophosphate (cAMP) signal and thereby tightly regulating β AR-stimulated contractility [7]. It has been postulated that this PDE4D5 recruitment to the agonist-activated cardiac β_2 AR poses a "brake" on the β_2 AR cAMP signaling's ability to stimulate contractility [6,7,13,15]. By contrast, agonist-promoted dissociation of PDE from the β_1 AR underlies the more "diffuse" and more powerful at stimulating contraction signaling of this β AR subtype [6,7]. Of note, cardiac β_2 AR signaling has been reported to become more "diffuse" and decompartmentalized, i.e. to adopt a β_1 AR-like signaling pattern, in a rat model of HF, which might underlie cardiac β_2 AR dysfunction in HF [17].

On the other hand, cardiac β_2 AR can switch its signaling from G_s protein-mediated to $G_{i/o}$ protein-mediated, which is believed to underlie its anti-apoptotic effects and is a feature cardiac β_1 ARs lack [18-24]. Finally, the interactions of the β_2 AR with the β arrs, which require prior receptor phosphorylation by GRKs, can have pleiotropic effects in cardiac myocytes, such as inhibition of apoptosis/promotion of survival by promoting extracellular signal-regulated kinase (ERK) signaling [25] and inhibition of inflammation by blocking the pro-inflammatory transcription factor nuclear factor-kappaB (NF- κ B) [26,27], a crucial mediator of major pro-inflammatory cytokine expression, such as tumor necrosis factor-alpha (TNF- α) and interleukin-1 and -6 (IL-1 & -6) [28-30]. These β arr-dependent signaling effects may also play some part in the

well known and described anti-apoptotic and other beneficial in post-MI HF effects of cardiac β_2 AR.

Cardiac GRK2 is a major negative regulator of β AR pro-contractile signaling [8-11]. By desensitizing both β_1 - and β_2 ARs, i.e. terminating their G protein-mediated signaling through cAMP, it dramatically reduces cardiac inotropic and adrenergic reserves, and since it is markedly elevated in HF, its blockade represents an attractive therapeutic strategy for heart disease treatment [8-11,31-35]. Given that GRK2 can block the pro-contractile and other beneficial signaling of cardiac β_2 AR in HF, and also that its action on β_2 AR induces recruitment of β arrs with all their aforementioned myriad effects on this receptor's signaling, we hypothesized, in the present study, that cardiac GRK2 blockade in vivo might enhance β_2 AR signaling post-MI. In order to study the effects of GRK2 blockade specifically on this subtype's signaling, without any interference by the qualitatively different β_1 AR signaling, we utilized the β_1 AR knockout (B1KO) mice [36], which we crossed with mice overexpressing the known GRK2 inhibitor mini-gene β ARKct (or GRK2ct) specifically in cardiac myocytes [32]. After breeding the offspring to homozygosity, we studied them both under normal conditions (i.e. healthy, sham-operated animals) and after surgically induced MI to induce HF. We found that GRK2 inhibition in vivo is absolutely necessary for the β_2 AR to be capable of increasing contractility. In addition, β_2 AR anti-apoptotic signaling post-MI is augmented by β ARKct, but only acutely.

Results

β ARKct restores cardiac β_2 AR-dependent pro-contractile signaling by reducing the interaction of PDE4D with the receptor

We developed the hybrid transgenic line β ARKct/B1KO by crossing the B1KO mice with the β ARKct transgenic mice, which express β ARKct only in cardiomyocytes. The β ARKct/B1KO's breed normally, without any gross abnormalities and present no overt cardiovascular or other phenotype (data not shown). Three-month-old male mice were chosen to undergo surgical MI in order to induce HF and were studied alongside age-matched male homozygous B1KO's (without β ARKct expression), which served as the control group.

Since GRK2 is a major (negative) regulator of cardiac β AR-dependent contractility in vivo, and the β_2 AR stimulates contractility only very weakly, we first examined the cardiac function parameters of these mice, both in sham and post-MI groups. Echocardiography revealed that the B1KO mice display significantly decreased ejection fraction compared to control wild type (WT) mice, both under normal conditions (sham groups) and at 4 weeks post-MI, as expected since the β_1 AR is the major β AR subtype in the heart stimulating contractility (Table 1) [8]. Notably, β ARKct overexpression led to significant augmentation of

Table 1 In vivo cardiac functional parameters

Parameter	WT-Sham	β ARKct-Sham	B1KO-Sham	β ARKct/ B1KO-Sham	WT-MI	β ARKct-MI	B1KO-MI	β ARKct/ B1KO-MI
Infarct size (% LV free wall)	N/A	N/A	N/A	N/A	41±1.2	42±2.2	40±2.6	43±3.0
FS (%)	41.4±1.3	48.3±2.8 [^]	36.7±2.6 [^]	41.3±2.4 [#]	18.6±2.2	23.7±1.3 ⁺	11.0±1.4 ⁺	19.0±2.4 [*]
EF (%)	72.0±1.0	78.0±2.0 [^]	67.0±2.4 [^]	72.0±2.0 [#]	37.5±3.0	45.3±2.4 ⁺	23.7±2.5 ⁺	38.5±4.2 [*]
Basal HR (min ⁻¹)	385±12	402±37	402±27	383±23	392±12	408±10	391±8	389±13
Basal LV +dP/dt _{max} (mm Hg/s)	4692±363	6835±637 [^]	3572±287 [^]	4576±375 [#]	3512±220	4900±172 ⁺	2576±155 ⁺	3549±231 [*]
Basal LV -dP/dt _{min} (mm Hg/s)	-4568±452	-6714±536 [^]	-3269±296 [^]	-4782±286 [#]	-3515±246	-4620±203 ⁺	-2592±233 ⁺	-3471±194 [*]
Max. Iso-HR (min ⁻¹)	557±36	515±40	405±28 [^]	393±14 [^]	509±24	581±17 ⁺	399±11 ⁺	413±20 ⁺
Max. Iso-LV +dP/dt _{max} (mm Hg/s)	8825±644	13547±476 [^]	3611±286 [^]	9041±528 [#]	5641±431	9419±601 ⁺	2639±139 ⁺	5628±419 [*]
Max. Iso-LV -dP/dt _{min} (mm Hg/s)	-7210±430	-8837±491 [^]	-3199±279 [^]	-6344±364 [#]	-5880±303	-6948±277 ⁺	-2605±251 ⁺	-5548±382 [*]

Cardiac parameter values of three-month-old wild type (WT, i.e. C57/B6), β ARKct, B1KO and β ARKct/B1KO mice measured at 4 weeks after MI or sham operation (Sham). LV +dP/dt_{max} maximal first derivative of LV pressure rise, LV -dP/dt_{min} minimal first derivative of LV pressure fall, HR heart rate, FS, fractional shortening, EF ejection fraction, LV Left ventricular, N/A Not applicable, Max. Iso dose: 333 ng/kg body weight. #, p<0.05, vs. B1KO-Sham; *, p<0.05, vs. B1KO-MI; ^, p<0.05, vs. WT-Sham; +, p<0.05, vs. WT-MI; n=7 mice/group. One-way ANOVA with Bonferroni test was performed among groups. Data are presented as mean ± SEM.

the ejection fraction of the B1KO mice, up to the levels of WT mice, again both in normal and in 4 week post-MI mice (Table 1), while, as already known from our studies in the past [10,11,32,33], β ARKct significantly augments contractility of the WT mice, as well (Table 1). Importantly, when the mice underwent in vivo cardiac catheterization to measure their hemodynamic responses to isoproterenol stimulation (a standard β AR full agonist), B1KO mice, remarkably, completely failed to show any increase in contractility (as measured by the +dP/dt_{max} LV pressure elevation parameter), even at the highest concentration of isoproterenol challenge (Max. Iso, Table 1). In contrast, the hybrid β ARKct/B1KO mice showed very good contractile responses to isoproterenol, both in the sham (healthy) conditions and in post-MI HF (Table 1). As expected, the other two mouse lines, i.e. WT and β ARKct, were responsive to β AR stimulation, with the HF animals in these groups showing somewhat reduced responses compared to their sham counterparts and the β ARKct line displaying much more robust responses compared to the WT group (Table 1). These results strongly indicate that cardiac GRK2 is a major opposing force for the β_2 AR pro-contractile function and only when its activity is blocked (e.g. in the presence of β ARKct) is the cardiac β_2 AR capable of promoting contractility in response to agonist stimulation.

To identify the main signaling mechanism underlying this dramatic effect of β ARKct on cardiac β_2 AR-dependent contractility, we examined the levels of PDE4D interaction with the β_2 AR in cardiac membranes of these mice in vivo. As shown in Figures 1A and 1B, the interaction of cardiac β_2 AR with both the PDE4D3 and -D5 isoforms is significantly reduced in β ARKct/

B1KO mouse hearts compared to control B1KO hearts, an effect that might enable β ARKct to enhance cardiac β_2 AR-dependent pro-contractile signaling in vivo.

β ARKct and cardiac β_2 AR-dependent anti-apoptotic/inflammatory signaling

Next, we examined the impact of β ARKct expression on the other major aspect of cardiac β_2 AR signaling, anti-apoptosis/cardiac survival. Post-MI β ARKct/B1KO mice display markedly better survival post-MI compared to their B1KO counterparts (Figure 2A). Kaplan-Meier survival curves indicated that at 4 weeks post-MI, a significant (~70%) of β ARKct/B1KO's are still alive, compared to only ~40% of B1KO's at the same time point post-MI (Figure 2A). In addition, cardiac apoptosis is found significantly decreased very early (at 24 hrs) post-MI in the β ARKct/B1KO hearts compared to control B1KO hearts (Figure 2B) but similar between the two groups at 4 weeks post-MI (Figure 2B), indicating that this reduction in post-MI apoptosis induced by β ARKct is short-lived. As for post-MI cardiac inflammation in the two animal groups, levels of the major pro-inflammatory cytokines TNF α (Figure 2C), IL-6 (Figure 2D) and IL-1 β (Figure 2E) are significantly increased in the hearts of β ARKct/B1KO mice, compared to control B1KO hearts at 24 hrs post-MI, indicating increased wound (infarct) healing-associated inflammation. By 4 weeks post-MI however, levels of all these three cytokines (TNF α , IL-6, IL-1 β) in β ARKct/B1KO hearts have returned to the levels of 24-hour post-MI B1KO hearts (data not shown), indicating that also the effect of β ARKct on post-MI inflammation is short-lived.

To identify potential signaling mechanisms underlying these effects of β ARKct on apoptosis and inflammation

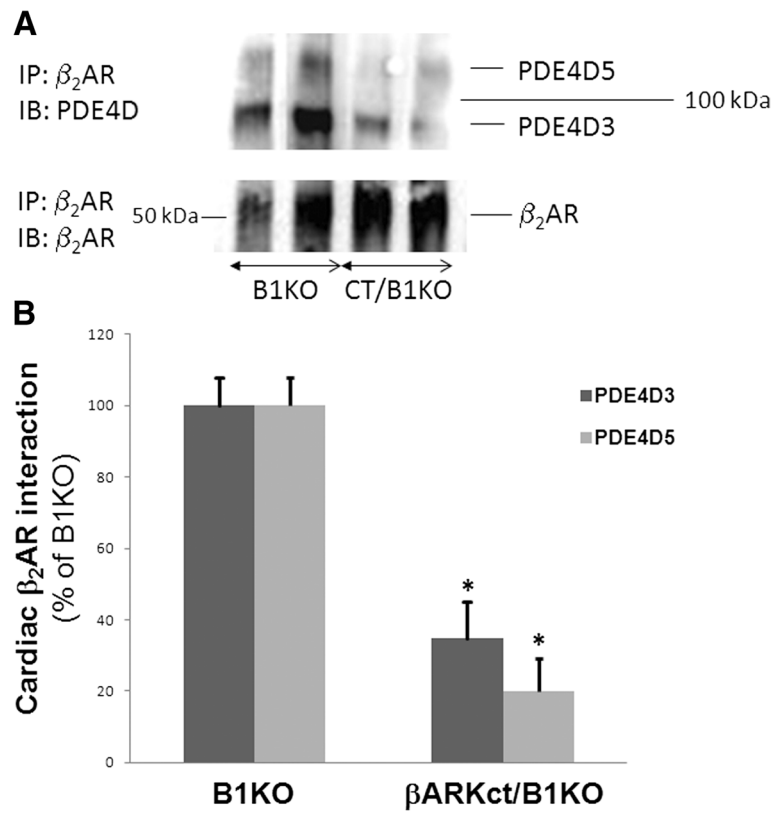


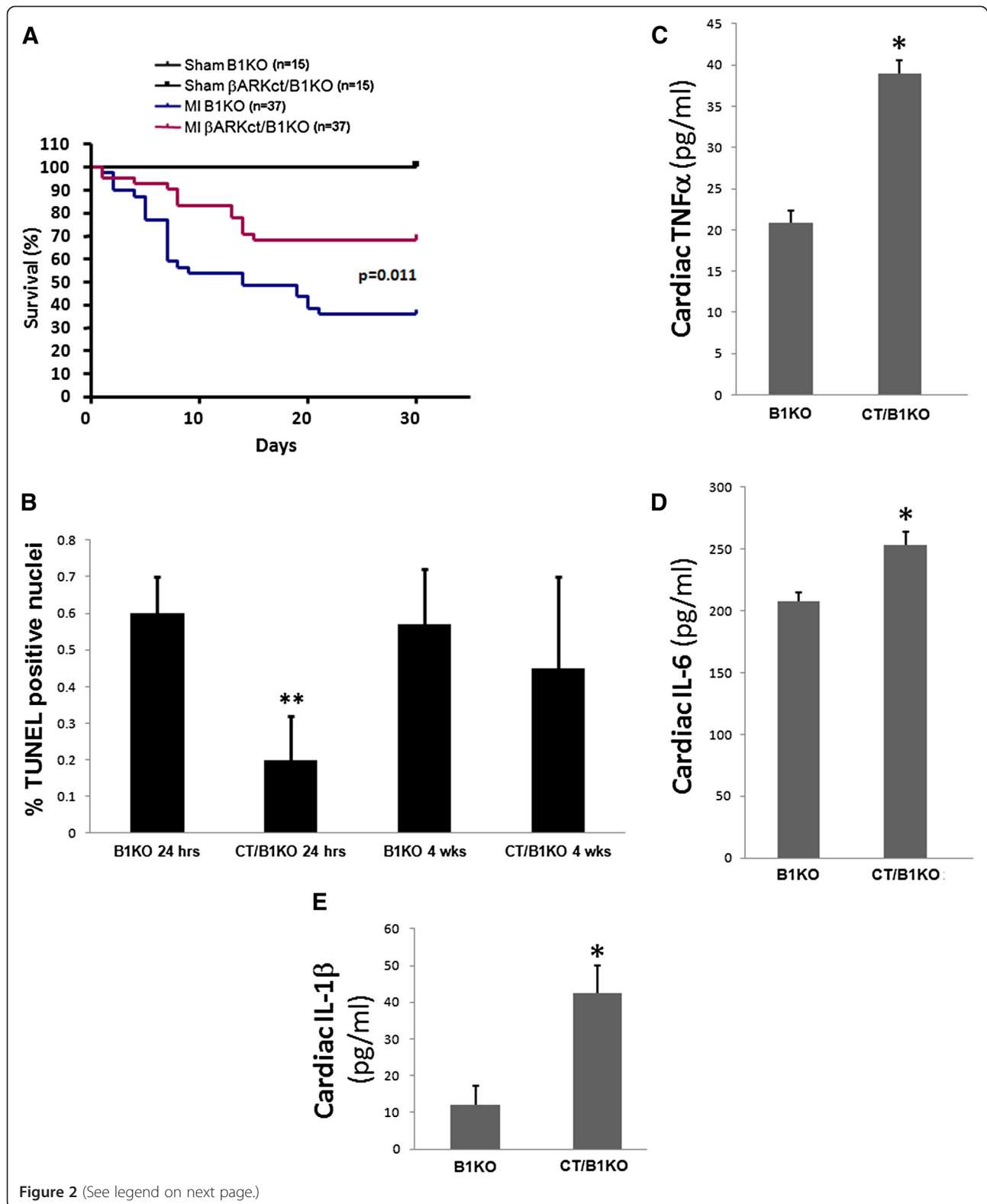
Figure 1 β_2AR -PDE4D interaction in the heart. Co-immunoprecipitation (co-IP) followed by western blotting in cardiac extracts from normal (sham) B1KO and $\beta ARKct/B1KO$ (CT/B1KO) mice to measure the β_2AR -PDE4D interaction in the heart. Representative immunoblots are shown in (A), and the amounts of the co-immunoprecipitated PDE4D isoforms, as measured by densitometry and normalized with the amount of β_2AR pulled down in the co-IP, are shown in (B). *, $p < 0.05$, vs. B1KO; $n = 4$ independent experiments (i.e. performed on 4 different hearts from each mouse line). IP: Immunoprecipitation, IB: Immunoblotting.

in post-MI B1KO hearts, we examined protein levels of the major anti-apoptotic mediator Bcl-2 [37] and levels of activation of the crucial pro-inflammatory transcription factor NF κ B. Bcl-2 was found significantly up-regulated in $\beta ARKct/B1KO$ hearts compared to control B1KO hearts at 24 hrs post-MI (Figures 3A and 3B), indicating enhanced cellular survival/inhibition of apoptosis. However, at 4 weeks post-MI, Bcl-2 protein was virtually undetectable in the hearts of both mouse lines (Figure 3C), which is consistent with the phenotypic finding of the short-lived inhibition of apoptosis in the heart by $\beta ARKct$ (Figure 2B). In addition, NF κ B activation appears also markedly elevated in $\beta ARKct/B1KO$ hearts compared to control B1KO hearts at 24 hrs post-MI (Figures 3A and 3B), indicating enhanced cardiac inflammation. For NF κ B to get activated, its inhibitory I κ B α subunit must be phosphorylated and subsequently targeted for proteasomal degradation to release the transcriptionally active subunits of the complex [28-30]. Thus, increased phosphorylation of I κ B α and decreased levels (increased degradation) of total I κ B α in the hybrid

transgenic hearts at 24 hrs post-MI (Figures 3A and 3B) suggest increased NF κ B activation compared to B1KO hearts. Finally, blotting for $\beta ARKct$ in these hearts confirmed the robust expression of this GRK2 inhibitor in the hearts of $\beta ARKct/B1KO$'s, which, of course, was absent from the hearts of B1KO mice (Figure 3A).

GRK5 and cardiac β_2AR -dependent signaling

Apart from GRK2, the other major cardiac GRK that can phosphorylate and desensitize β_2AR s, and thus oppose β_2AR pro-contractile and anti-apoptotic signaling, is GRK5 [8-10]. As shown in Figure 3D, cardiac post-MI GRK5 levels are initially (at 24 hrs post-MI) similar between the two groups, as is the case also in the healthy, sham-operated groups (data not shown). By 4 weeks post-MI however, a significant up-regulation (~2-fold increase) of GRK5 is observed in $\beta ARKct/B1KO$ hearts compared to control B1KO hearts (Figure 3D), indicating that GRK2 inhibition with $\beta ARKct$ leads to a compensatory up-regulation of GRK5 over time.



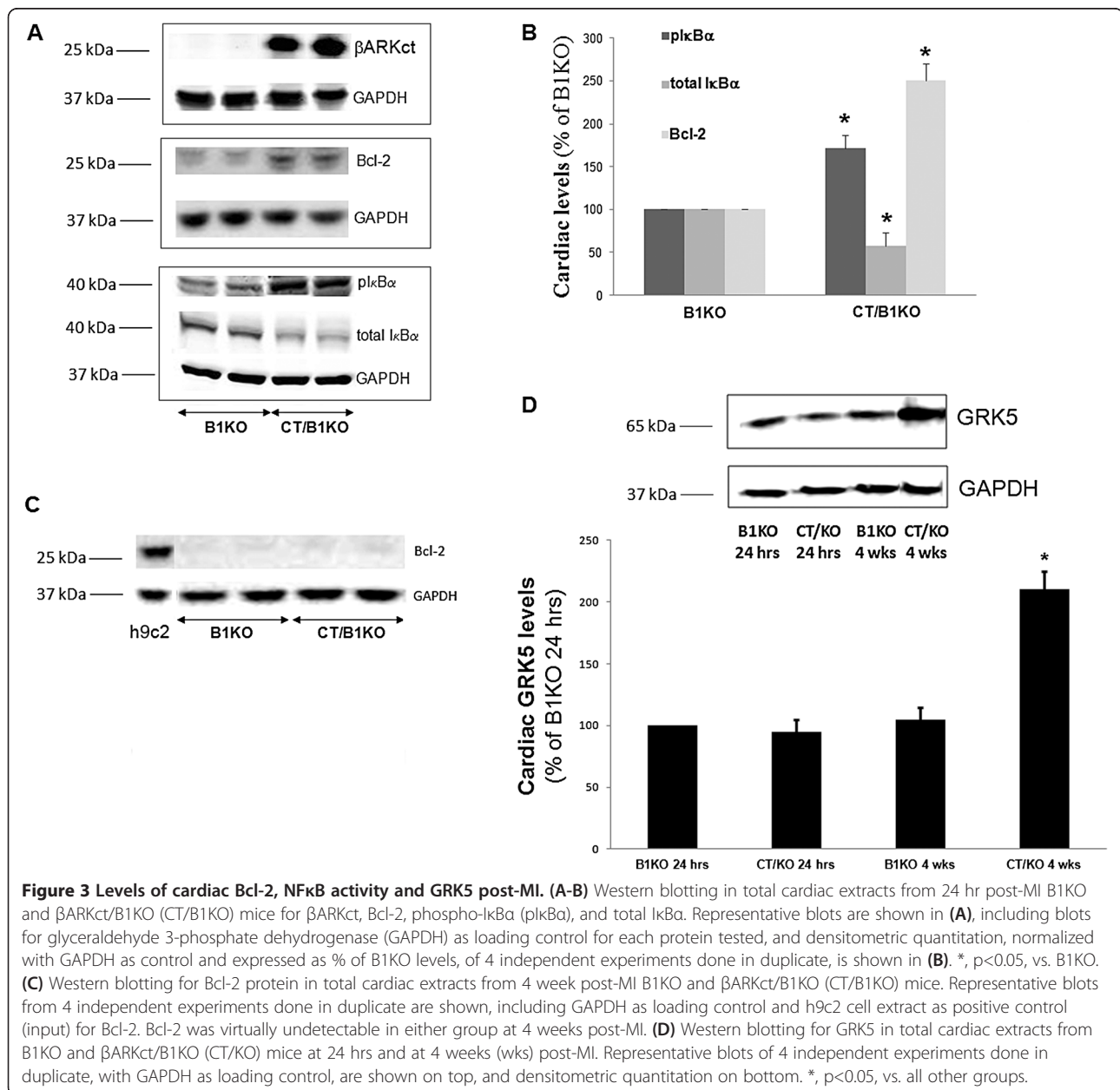
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Figure 2 Survival, cardiac apoptosis and inflammation post-MI. (A) Kaplan-Meier survival curves of the 4 groups of mice of the study: sham-operated (Sham) and post-MI (MI) B1KO and β ARKct/B1KO mice. $p=0.011$ between MI B1KO and MI β ARKct/B1KO; $n=15$ mice/group for sham, 37 mice/group for MI mice. (B) Apoptotic cell death at 24 hrs and at 4 weeks (wks) post-MI in the two transgenic (B1KO and CT/B1KO) lines, as measured by TUNEL performed in the border zone of the infarct. No difference in rate of apoptosis in the remote zone at either post-MI time point was found (data not shown). **, $p<0.05$, vs. B1KO 24 hrs, $n=6$ mice/group. (C-E) Levels of pro-inflammatory cytokines TNF α (C), IL-6 (D), and IL-1 β (E), measured via ELISA in serum of intra-cardiac blood from B1KO and β ARKct/B1KO (CT/B1KO) mice at 24 hrs post-MI. *, $p<0.05$, $n=5$ mice/group.

Discussion

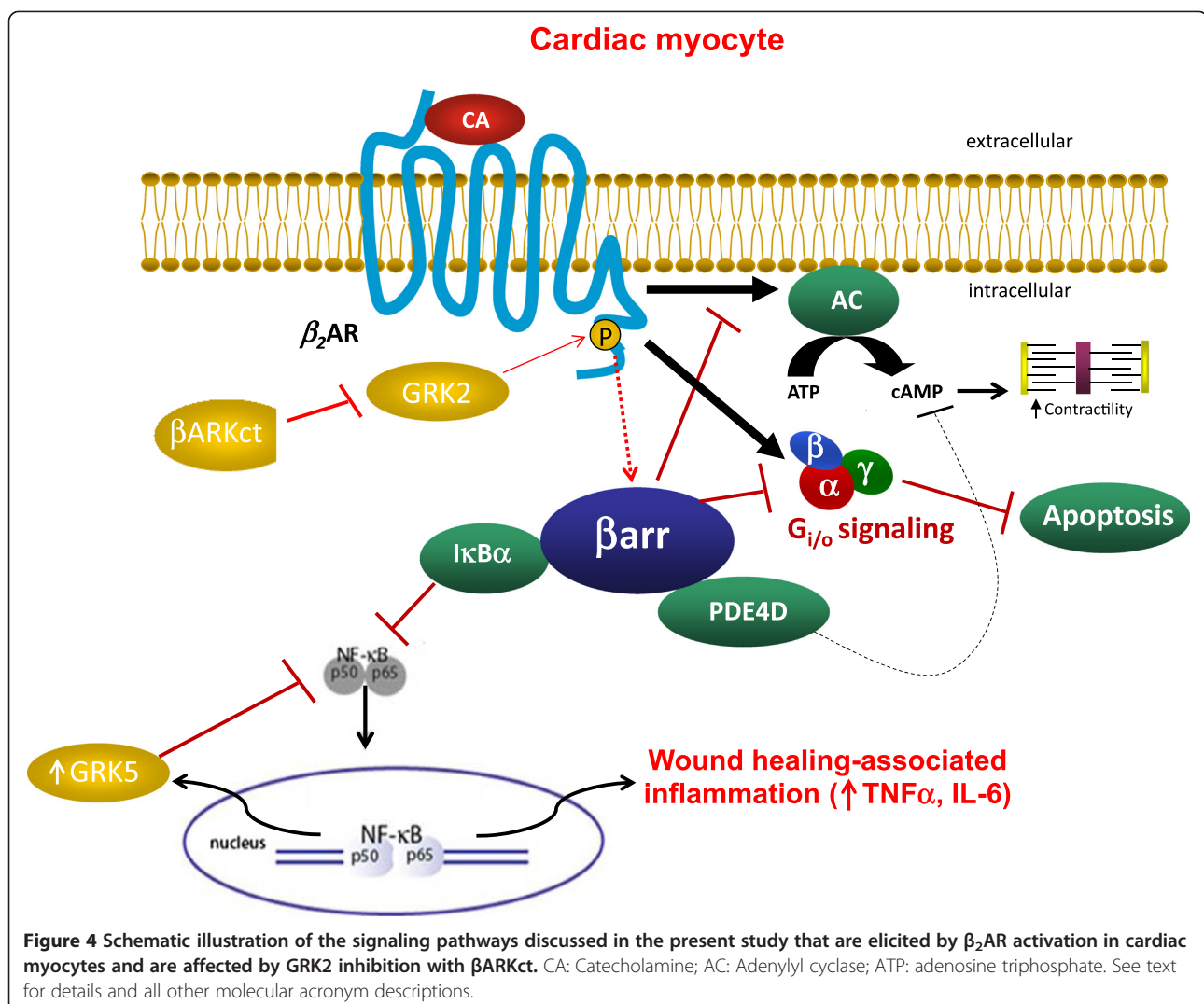
The present study reports for the first time, to our knowledge, that cardiac GRK2 is an endogenous “stumbling block” that normally prevents β_2 AR signaling from stimulating contractility, mainly because it promotes

association of this β AR subtype with PDE4D in the heart, a major molecular “brake” on cardiac β_2 AR-dependent contractility [6,7,15]. Thus, only when cardiac GRK2 is blocked (e.g. with β ARKct) is the β_2 AR capable of promoting cardiac contractility. Obviously, several



signaling mechanisms/pathways are at play, the present study has identified the following two: 1) β ARKct, by blocking GRK2, reduces the uncoupling of β_2 AR with the classical pro-contractile G_s protein-adenylyl cyclase-cAMP-PKA signaling pathway (Figure 4), and 2) GRK2 blockade reduces the interaction of β_2 AR with β arrs, which scaffold on themselves various isoforms of PDE4D (mainly PDE4D3 and PDE4D5) (Figure 4). PDE4D causes degradation of the local cAMP signals produced by activated β_2 AR, which are essential for stimulation of contractility, and thus it weakens these pro-contractile signals hampering β_2 AR-stimulated contractility [6,7,15]. By indirectly reducing the β arr-PDE4D interaction with the cardiac β_2 AR, β ARKct releases the “brake” PDE4D poses on this receptor’s pro-contractile signaling and enhances its capacity to stimulate contractility (Figure 4).

Since the other major beneficial effect of cardiac β_2 AR signaling in vivo is inhibition of apoptosis (promotion of survival), we also tested the effects of cardiac GRK2 blockade in vivo on this aspect of β_2 AR signaling in the context of post-MI HF progression. We found that early on after MI, cardiac GRK2 blockade with β ARKct also dramatically augments β_2 AR anti-apoptotic signaling, as well as its pro-infarct healing inflammatory signaling, in the heart. This results in significant reduction in all-cause mortality (marked increase in animal survival in the first 4 weeks post-MI), and reduced cellular apoptosis in the post-MI heart, compared to B1KO mice with unopposed cardiac GRK2 activity. Thus, β ARKct enhances not only cardiac contractility, but also cardiac survival stimulated by the β_2 AR, which further reinforces its validity as an attractive therapeutic strategy to potentiate cardiac β_2 AR signaling and function in post-MI



HF. Of course, enhancement of the anti-apoptotic signaling of other cardio-protective receptors that are also GRK2 substrates by β ARKct cannot be ruled out and is, in fact, quite likely to have contributed to the observed cardiac apoptosis phenotype of β ARKct/B1KO mice. However, β ARKct's cardio-protective and anti-apoptotic effects have been shown to be β_2 AR-dependent, since selective blockade of this receptor in cardiac myocytes abolishes β ARKct-mediated anti-apoptotic effects [38]. On the other hand, effects of β ARKct on β_2 AR-dependent pro-angiogenic signaling, which plays an important role in peri-infarct HF progression [39], cannot be ruled out either. Nevertheless, it becomes quite clear from our current data that β ARKct augments β_2 AR contractile function without negatively affecting its anti-apoptotic one, but rather actually preserving and further enhancing this β_2 AR function, as well.

However, this augmentation of anti-apoptotic signaling is short-lived: by 4 weeks post-MI, cardiac cellular apoptosis has returned to the 24-hour post-MI B1KO heart levels. This might be related to the nature of cardiac β_2 AR pro-survival signaling; β_2 AR can have remarkably different effects in the heart depending on its expression levels and on time [40,41]. Cardiac β_2 AR is known to be beneficial (i.e. promoting survival) at low levels of overexpression and in the first few months of life in mice, but when overexpressed at extremely high levels in murine hearts or later on in the mouse's life, these animals do not survive and die of severe cardiac complications [40]. Mechanistically, cardiac β_2 AR anti-apoptotic signaling is known to proceed mainly through the $G_{i/o}$ protein signaling pathway [20-23], to which it is capable of switching following its phosphorylation by PKA [42]. GRK2 blockade by β ARKct can increase this signaling by a) decreasing the pathway's β arr-mediated desensitization, i.e. increasing the coupling of $G_{i/o}$ proteins with the β_2 AR, and b) by increasing the PKA-dependent switching of β_2 AR signaling from G_s to $G_{i/o}$ proteins thanks to the increase of β_2 AR signaling via the G_s protein-cAMP-PKA (the pro-contractile) pathway it also causes, discussed above (Figure 4). With regards to the pro-inflammatory signaling of cardiac β_2 AR, β arrs are known to scaffold and stabilize the inhibitory I κ B α subunit of NF κ B, thereby prohibiting NF κ B activation [26,27]. GRK2 blockade with β ARKct decreases β arr interaction with the β_2 AR thereby "releasing" the inhibitory effect of β arrs on NF κ B activation (Figure 4). Thus, NF κ B activation and the subsequent pro-inflammatory cytokine production are enhanced (Figure 4). Indeed, NF κ B activation and inflammatory cytokine levels were found significantly elevated in β ARKct/B1KO hearts compared to B1KO hearts without GRK2 inhibition at 24 hrs post-MI.

Meanwhile however, β ARKct also causes upregulation of the other major cardiac GRK, GRK5, in the first few weeks post-MI. This is also probably due to the enhanced NF κ B activation (Figure 4), since NF κ B can cause

upregulation of GRK5 in cardiomyocytes [43]. Importantly, and given that GRK5 elevation is generally considered detrimental for the heart [8-10], this finding might explain, at least in part, the aforementioned switching of cardiac β_2 AR signaling from beneficial (anti-apoptotic) early in life of transgenic mice or at low levels of receptor expression to detrimental (pro-apoptotic) later on in the lifespan of these mice or at very high levels of cardiac β_2 AR overexpression [41]. Of note, GRK5 has also been reported to bind (via its non-catalytic, N-terminal domain) to, and stabilize I κ B α , thereby inhibiting NF κ B activity in several tissues, including the heart [44]. Therefore, our present findings strongly indicate that a negative feedback loop might exist in the heart, in which NF κ B induces GRK5 expression, and GRK5 can subsequently suppress NF κ B activation (Figure 4).

Conclusions

In summary, the present study reports that cardiac GRK2 inhibition with β ARKct in vivo is absolutely essential for the cardiac β_2 AR subtype's pro-contractile function, all the while preserving and augmenting this receptor's beneficial anti-apoptotic/pro-survival and pro-infarct healing signaling pathways post-MI, early on after the cardiac insult. However, the effects of β ARKct on the latter signaling modalities are transient due (in part) to compensatory elevation of cardiac GRK5 over time.

Methods

Experimental animals and surgical procedures

The animals in this study were handled according to animal welfare regulations and protocols approved by the authors' Institutional Review Boards. Genetically engineered, 8- to 12-wk-old β_1 AR KO (B1KO) (on C57/B6 background) [36] and the offspring of their cross with Mini-27 mice, expressing the β ARKct (or GRK2ct) transgene under the alpha-myosin heavy chain gene promoter [32], were used for this study. Mice were anesthetized with a mixture of ketamine (100 mg/kg) and xylazine (2.5 mg/kg). Animals were placed in the supine position on a heated operation board and a midline cervical incision was made to expose the trachea. Following successful endotracheal intubation, the cannula was connected to a rodent ventilator. The entire left ventricle (i.e. both infarct and non-infarct zones) were used for subsequent histological and biochemical assays. Myocardial infarction was performed as previously described [35].

Echocardiography & in vivo hemodynamics

Transthoracic echocardiography was performed with a linear 30-MHz transducer (VeVo 770 High Resolution Imaging System, VisualSonics, Toronto, ON, Canada), as

described [35]. In vivo hemodynamic analysis was also performed as previously described [35].

In situ TUNEL staining

Heart specimens were fixed with 10% neutral buffered formalin, embedded in paraffin, and sectioned at 5- μ m thickness. DNA fragmentation was detected in situ in deparaffinized sections using the ApopTag Kit (Intergene) and according to manufacturer's instructions, as described previously [45]. The total number of nuclei was determined by manual counting of DAPI-stained nuclei in six random fields per section. All terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL)-positive nuclei were counted in each section.

Co-immunoprecipitation and western blotting

Cardiac extracts were prepared in 20 mM Tris pH 7.4, 137 mM NaCl, 1% Nonidet P-40, 20% glycerol, 10 mM PMSE, 1 mM Na₃VO₄, 10 mM NaF, 2.5 μ g/ml aprotinin, and 2.5 μ g/ml leupeptin. Protein concentration was then determined and equal amounts of protein per sample were loaded on SDS-PAGE gels for electrophoretic separation, as described previously [46]. For β_2 AR-PDE4D co-immunoprecipitation experiments, β_2 AR was immunoprecipitated with an anti-mouse β_2 AR antibody (sc-9042, Santa Cruz), immobilized on Protein A-sepharose beads (Invitrogen), prior to SDS-PAGE/western blotting. Total I κ B α and phospho-I κ B α were detected by using anti-I κ B α (sc-1643, Santa Cruz) and anti-phosphoI κ B α at Ser-32 (sc-7977, Santa Cruz) antibodies, Bcl-2, GRK5, GRK2/ β ARKct and GAPDH, with antibodies sc-492, sc-565, sc-562, and sc-25778, respectively (all from Santa Cruz), and PDE4D (various isoforms) with the PD4-401AP antibody (FabGennix). Immunoblots were revealed by enhanced chemiluminescence (ECL, Amersham Biosciences) and visualized in the FluorChem E Digital Darkroom (Cell Biosciences). Densitometry was performed with the AlphaView software (Cell Biosciences) in the linear range of signal detection (on non-saturated bands).

Cytokine measurements via ELISA

Pro-inflammatory cytokines TNF α , IL-6 and IL-1 β were measured in serum obtained from left ventricular blood, immediately prior to heart excision and animal euthanizing, via multiplexed ELISA, as described [47,48]. The assay was performed using the Mouse Cytokine ELISA Profiling Kit (EA-1091, Signosis), according to the manufacturer's instructions.

Statistical analyses

Data are generally expressed as mean \pm SEM. Unpaired 2-tailed Student's *t* test and one- or two-way ANOVA with Bonferroni test were generally performed for statistical

comparisons, unless otherwise indicated. For all tests, a *p* value of <0.05 was generally considered to be significant.

Abbreviations

β AR: Beta-adrenergic receptor; B1KO: β_1 AR knockout; GRK: G protein-coupled receptor kinase; β ARKct: Beta-adrenergic receptor kinase (GRK2) carboxyl terminal fragment; β arr: Beta-arrestin; PDE: Phosphodiesterase; MI: Myocardial infarction; HF: Heart failure; NF κ B: Nuclear factor-kappaB; I κ B α : Inhibitor of nuclear factor-kappaB alpha subunit; cAMP: 3'-5' adenosine monophosphate (cyclic adenosine monophosphate); WT: Wild type; PKA: Protein kinase A; G_s: Stimulatory G protein; G_{i/o}: Inhibitory or other G protein; TNF α : Tumor necrosis factor alpha; IL: Interleukin; TUNEL: Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling; Bcl-2: B-cell lymphoma 2; LV: Left ventricular; ELISA: Enzyme-linked immunosorbent assay.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

NCS, XV, AS, GR, AC, DLic, CDL, and EG performed research. DLeos assisted with writing of the paper. WJK supervised the project, provided funding for the research and assisted with writing of the paper. AL conceived and supervised the project, designed research, provided funding for it, and wrote the paper. All authors have read and approved the final manuscript.

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References

1. Tamargo J, López-Sendón J: **Novel therapeutic targets for the treatment of heart failure.** *Nat Rev Drug Disc* 2011, **10**:536–555.
2. Xiang Y, Kobilka BK: **Myocyte adrenoceptor signaling pathways.** *Science* 2003, **300**:1530–1532.
3. Xiao RP, Zhu W, Zheng M, Chakir K, Bond R, Lakatta EG, Cheng H: **Subtype-specific beta-adrenoceptor signaling pathways in the heart and their potential clinical implications.** *Trends Pharmacol Sci* 2004, **25**:358–365.
4. Devic E, Xiang Y, Gould D, Kobilka B: **Beta-adrenergic receptor subtype-specific signaling in cardiac myocytes from beta(1) and beta(2) adrenoceptor knockout mice.** *Mol Pharmacol* 2001, **60**:577–583.
5. Bernstein D, Fajardo G, Zhao M, Urashima T, Powers J, Berry G, Kobilka BK: **Differential cardioprotective/cardiotoxic effects mediated by beta-adrenergic receptor subtypes.** *Am J Physiol Heart Circ Physiol* 2005, **289**:H2441–H2449.
6. Xiang Y, Naro F, Zoudilova M, Jin SL, Conti M, Kobilka B: **Phosphodiesterase 4D is required for beta2 adrenoceptor subtype-specific signaling in cardiac myocytes.** *Proc Natl Acad Sci USA* 2005, **102**:909–914.
7. Richter W, Day P, Agrawal R, Bruss MD, Granier S, Wang YL, Rasmussen SG, Horner K, Wang P, Lei T, Patterson AJ, Kobilka B, Conti M: **Signaling from beta1- and beta2-adrenergic receptors is defined by differential interactions with PDE4.** *EMBO J* 2008, **27**:384–393.
8. Rockman HA, Koch WJ, Lefkowitz RJ: **Seven-transmembrane-spanning receptors and heart function.** *Nature* 2002, **415**:206–212.
9. Rengo G, Lympopoulos A, Koch WJ: **Future g protein-coupled receptor targets for treatment of heart failure.** *Curr Treat Options Cardiovasc Med* 2009, **11**:328–338.

10. Belmonte SL, Blaxall BC: **G protein coupled receptor kinases as therapeutic targets in cardiovascular disease.** *Circ Res* 2011, **109**:309–319.
11. Rengo G, Lympopoulos A, Leosco D, Koch WJ: **GRK2 as a novel gene therapy target in heart failure.** *J Mol Cell Cardiol* 2011, **50**:785–792.
12. Baillie GS, Sood A, McPhee I, Gall I, Perry SJ, Lefkowitz RJ, Houslay MD: **beta-Arrestin-mediated PDE4 cAMP phosphodiesterase recruitment regulates beta-adrenoceptor switching from G_s to G_i.** *Proc Natl Acad Sci USA* 2003, **100**:940–945.
13. Houslay MD, Adams DR: **PDE4 cAMP phosphodiesterases: modular enzymes that orchestrate signalling cross-talk, desensitization and compartmentalization.** *Biochem J* 2003, **370**:1–18.
14. De Arcangelis V, Liu R, Soto D, Xiang Y: **Differential association of phosphodiesterase 4D isoforms with beta2-adrenoceptor in cardiac myocytes.** *J Biol Chem* 2009, **284**:33824–33832.
15. Fischmeister R, Castro LR, Abi-Gerges A, Rochais F, Jurevicius J, Leroy J, Vandecasteele G: **Compartmentation of cyclic nucleotide signaling in the heart: the role of cyclic nucleotide phosphodiesterases.** *Circ Res* 2006, **99**:816–828.
16. Conti M, Beavo J: **Biochemistry and physiology of cyclic nucleotide phosphodiesterases: essential components in cyclic nucleotide signaling.** *Annu Rev Biochem* 2007, **76**:481–511.
17. Nikolaeve VO, Moshkov A, Lyon AR, Miragoli M, Novak P, Paur H, Lohse MJ, Korchev YE, Harding SE, Gorelik J: **Beta2-adrenergic receptor redistribution in heart failure changes cAMP compartmentation.** *Science* 2010, **327**:1653–1657.
18. Chesley A, Lundberg MS, Asai T, Xiao RP, Ohtani S, Lakatta EG, Crow MT: **The beta(2)-adrenergic receptor delivers an antiapoptotic signal to cardiac myocytes through G(i)-dependent coupling to phosphatidylinositol 3'-kinase.** *Circ Res* 2000, **87**:1172–1179.
19. Patterson AJ, Zhu W, Chow A, Agrawal R, Kosek J, Xiao RP, Kobilka B: **Protecting the myocardium: a role for the beta2 adrenergic receptor in the heart.** *Crit Care Med* 2004, **32**:1041–1048.
20. Xiao RP, Avdonin P, Zhou YY, Cheng H, Akhter SA, Eschenhagen T, Lefkowitz RJ, Koch WJ, Lakatta EG: **Coupling of beta2-adrenoceptor to Gi proteins and its physiological relevance in murine cardiac myocytes.** *Circ Res* 1999, **84**:43–52.
21. Zhu W, Zeng X, Zheng M, Xiao RP: **The enigma of beta2-adrenergic receptor Gi signaling in the heart: the good, the bad, and the ugly.** *Circ Res* 2005, **97**:507–509.
22. Hasseldine AR, Harper EA, Black JW: **Cardiac-specific overexpression of human beta2 adrenoceptors in mice exposes coupling to both G_s and G_i proteins.** *Br J Pharmacol* 2003, **138**:1358–1366.
23. Communal C, Singh K, Sawyer DB, Colucci WS: **Opposing effects of beta (1)- and beta(2)-adrenergic receptors on cardiac myocyte apoptosis: role of a pertussis toxin-sensitive G protein.** *Circulation* 1999, **100**:2210–2212.
24. Zhu WZ, Zheng M, Koch WJ, Lefkowitz RJ, Kobilka BK, Xiao RP: **Dual modulation of cell survival and cell death by beta(2)-adrenergic signaling in adult mouse cardiac myocytes.** *Proc Natl Acad Sci USA* 2001, **98**:1607–1612.
25. DeWire SM, Ahn S, Lefkowitz RJ, Shenoy SK: **Beta-arrestins and cell signaling.** *Annu Rev Physiol* 2007, **69**:483–510.
26. Witherow DS, Garrison TR, Miller WE, Lefkowitz RJ: **beta-Arrestin inhibits NF-kappaB activity by means of its interaction with the NF-kappaB inhibitor I-kappaBalpha.** *Proc Natl Acad Sci USA* 2004, **101**:8603–8607.
27. Gao H, Sun Y, Wu Y, Luan B, Wang Y, Qu B, Pei G: **Identification of beta-arrestin2 as a G protein-coupled receptor-stimulated regulator of NF-kappaB pathways.** *Mol Cell* 2004, **14**:303–317.
28. Valen G, Yan ZQ, Hansson GK: **Nuclear factor kappa-B and the heart.** *J Am Coll Cardiol* 2001, **38**:307–314.
29. Valen G: **Signal transduction through nuclear factor kappa B in ischemia-reperfusion and heart failure.** *Basic Res Cardiol* 2004, **99**:1–7.
30. Rothwarf DM, Karin M: **The NF-kappa B activation pathway: a paradigm in information transfer from membrane to nucleus.** *Sci STKE* 1999, **1999**:RE1.
31. Ungerer M, Böhm M, Elce JS, Erdmann E, Lohse MJ: **Altered expression of beta-adrenergic receptor kinase and beta 1-adrenergic receptors in the failing human heart.** *Circulation* 1993, **87**:454–463.
32. Koch WJ, Rockman HA, Samama P, Hamilton RA, Bond RA, Milano CA, Lefkowitz RJ: **Cardiac function in mice overexpressing the beta-adrenergic receptor kinase or a beta ARK inhibitor.** *Science* 1995, **268**:1350–1353.
33. Rockman HA, Choi DJ, Akhter SA, Jaber M, Giros B, Lefkowitz RJ, Caron MG, Koch WJ: **Control of myocardial contractile function by the level of beta-adrenergic receptor kinase 1 in gene-targeted mice.** *J Biol Chem* 1998, **273**:18180–18184.
34. Matkovich SJ, Diwan A, Klanke JL, Hammer DJ, Marreez Y, Odley AM, Brunskill EW, Koch WJ, Schwartz RJ, Dorn GW 2nd: **Cardiac-specific ablation of G-protein receptor kinase 2 redefines its roles in heart development and beta-adrenergic signaling.** *Circ Res* 2006, **99**:966–1003.
35. Raake PW, Vinge LE, Gao E, Boucher M, Rengo G, Chen X, DeGeorge BR Jr, Matkovich S, Houser SR, Most P, Eckhart AD, Dorn GW 2nd, Koch WJ: **G protein-coupled receptor kinase 2 ablation in cardiac myocytes before or after myocardial infarction prevents heart failure.** *Circ Res* 2008, **103**:413–422.
36. Rohrer DK, Desai KH, Jasper JR, Stevens ME, Regula DP Jr, Barsh GS, Bernstein D, Kobilka BK: **Targeted disruption of the mouse beta1-adrenergic receptor gene: developmental and cardiovascular effects.** *Proc Natl Acad Sci USA* 1996, **93**:7375–7380.
37. Whelan RS, Kaplinskiy V, Kitsis RN: **Cell death in the pathogenesis of heart disease: mechanisms and significance.** *Annu Rev Physiol* 2010, **72**:19–44.
38. Brinks H, Boucher M, Gao E, Chuprun JK, Pesant S, Raake PW, Huang ZM, Wang X, Qiu G, Gumpert A, Harris DM, Eckhart AD, Most P, Koch WJ: **Level of G protein-coupled receptor kinase-2 determines myocardial ischemia/reperfusion injury via pro- and anti-apoptotic mechanisms.** *Circ Res* 2010, **107**:1140–1149.
39. Santulli G, Cipolletta E, Sorriento D, Del Giudice C, Anastasio A, Monaco S, Maione AS, Condorelli G, Puca A, Trimarco B, Illario M, Iaccarino G: **CaMK4 gene deletion induces hypertension.** *J Am Heart Assoc* 2012, **1**:e001081.
40. Dorn GW 2nd, Tepe NM, Lorenz JN, Koch WJ, Liggett SB: **Low- and high-level transgenic expression of beta2-adrenergic receptors differentially affect cardiac hypertrophy and function in Galphaq-overexpressing mice.** *Proc Natl Acad Sci USA* 1999, **96**:6400–6405.
41. Liggett SB, Tepe NM, Lorenz JN, Canning AM, Jantz TD, Mitarai S, Yatani A, Dorn GW 2nd: **Early and delayed consequences of beta(2)-adrenergic receptor overexpression in mouse hearts: critical role for expression level.** *Circulation* 2000, **101**:1707–1714.
42. Daaka Y, Luttrell LM, Lefkowitz RJ: **Switching of the coupling of the beta2-adrenergic receptor to different G proteins by protein kinase A.** *Nature* 1997, **390**:88–91.
43. Islam KN, Koch WJ: **Involvement of nuclear factor kappa B (NF-kappaB) signaling pathway in regulation of cardiac G protein-coupled receptor kinase 5 (GRK5) expression.** *J Biol Chem* 2012, **287**:12771–12778.
44. Sorriento D, Santulli G, Fusco A, Anastasio A, Trimarco B, Iaccarino G: **Intracardiac injection of AdGRK5-NT reduces left ventricular hypertrophy by inhibiting NF-kappaB-dependent hypertrophic gene expression.** *Hypertension* 2010, **56**:696–704.
45. Yoo B, Lemaire A, Mangmool S, Wolf MJ, Curcio A, Mao L, Rockman HA: **Beta1-adrenergic receptors stimulate cardiac contractility and CaMKII activation in vivo and enhance cardiac dysfunction following myocardial infarction.** *Am J Physiol Heart Circ Physiol* 2009, **297**:H1377–H13786.
46. Lympopoulos A, Rengo G, Funakoshi H, Eckhart AD, Koch WJ: **Adrenal GRK2 upregulation mediates sympathetic overdrive in heart failure.** *Nat Med* 2007, **13**:315–323.
47. Scott NJ, Cameron VA, Raudsepp S, Lewis LK, Simpson ER, Richards AM, Ellmers LJ: **Generation and characterization of a mouse model of the metabolic syndrome: apolipoprotein E and aromatase double knockout mice.** *Am J Physiol Endocrinol Metab* 2012, **302**:E576–E584.
48. Hu Y, Zhang H, Lu Y, Bai H, Xu Y, Zhu X, Zhou R, Ben J, Xu Y, Chen Q: **Class A scavenger receptor attenuates myocardial infarction-induced cardiomyocyte necrosis through suppressing M1 macrophage subset polarization.** *Basic Res Cardiol* 2011, **106**:1311–1328.

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