Supplemental Material

Generation of Transgenic Mice HA-Flag-tagged human β_2AR mutants lacking either the putative GRK phosphorylation sites (GRK-) or the putative PKA phosphorylation sites (PKA-) were subcloned into a vector downstream of myosin heavy Chain (MHC) gene promoter and upstream of the SV40 polyadenylation site (Fig. 2A&B). The GRK-β_2AR mutant was generated by mutating the serine/threonine amino acids residues in the carboxyl tail to alanine or glycine at positions 345,346, 355, 356, 360, 364, 384, 393, 396, 401, 407, 408, 411 [1] and the PKA- β_2AR mutant was generated by mutating the serine residues in the third intracellular loop to alanine at positions 261, 262, 345 and 346 [2]. The pBluescript II vector harbors a 5.5-kb murine α-MHC promoter (pMHC) and a SV-40 polyadenyltion sequence (a gift from Yibin Wang, University of California Los Angeles). For convenient cloning of GRK- and PKA- mutant β_2AR, a polylinker containing more sites of restriction endonucleases were inserted between Hind III and EcoR V. As a result, Mlu II, Afl II and Nhe I were introduced in the multiple clone sites by the polylinker sequence of AAG CTT ACG CGT GCT CTT AAG AGG CTA GCT AGC TAG GAT ATC. GRK- and PKA- mutated β_2AR cDNA sequences with N-terminal HA-Flag tags were amplified from pCDNA3-GRK and PBK-CMV-PKA using the primers of HA forward/GRK reverse and HA forward/PKA reverse respectively, and cloned into pBluescript II-MHC by Hind III/Nhe I. The primer sequences are: HA forward: atg ctc AAG CTT atg aag acc atc atc gcc ctg; PKA reverse: agt gca GCT AGC CAG CAG TGA GTC ATT TGT ACT AC; and GRK2 reverse: agt gca GCT AGC CAG CAG TGC GTC ATT TGC ACC AC. The expression cassettes (pMHC- β_2AR-PolyA) were released by Xho I/Not I from endotoxin-free prepared plasmid, Purified by agarose gel and injected into the fertilized mouse oocytes. Transgenic founders were identified by Southern blot analysis of tail DNA using the SV40 poly(A) as a probe. Transgenic founder mice were backcrossed to C57BL/6 mice for at least 7 generations before being used in experiments. Mice were screened by PCR with sense primer 5'-gac ctc tga cag aga agc agg c-3', located in the MHC (5264-5285), and an antisense primer, 5'-ggt acc agt gca tct gaa tgg g-3', located in β_2AR coding sequence (502-530). The product size is 810 bp. Transgenic mice with cardiac-specific overexpression of WT human β_2AR or its PKA- or GRK2- mutant were named WT TG, PKA- TG or GRK- TG mice, respectively. WT TG mice were imported from Dr. Gerald Dorn's lab. The founder of WT-β_2-AR TG is FVB background. The founder and its offspring bred with C57 background mice more than 7 generations. The offspring of 7th generation were used for experiments. Other mice strains are C57 background.

Animal models We used NTG, WT TG, PKA- TG, GRK- TG mice and GRK2 TG mice or their littermate controls (negative genotyping) at 12-16 weeks of age. Pressure overload was produced by transverse aortic constriction (TAC). Mice were anesthetized with isoflurane (2.5%) and pressure overload was produced by TAC as previously described[3]. Mice were placed supine on a heating pad (37%). A horizontal skin incision 0.5-1.0 cm was made at the level of suprasternal notch. To allow visualization of the aortic arch, a 2-3 mm longitudinal cut was made in the proximal portion of the sternum. A 6.00 silk suture was passed under the aorta. A 26-gauge needle was placed next to the aortic arch, and suture was tied around the needle and the aorta. After ligation, the needle was quickly removed. The chest was closed and the animal was allowed to recover after anesthesia. Doppler velocity was measured in the right and the left carotid arteries (RCA

and LCA) and RCA/LCA velocity ratio was calculated to ensure that TAC produced equal aortic pressure gradient in all experimental groups. Acute mortality within 24 hour after operation is not included in survival curves. National Institute of Aging Animal Care and Use Committee approved all animal experimental protocols.

Echocardiography In vivo cardiac morphology and function were assessed on anesthetized (2.0 isoflurane) mice by transthoraic echocardiography (Vevo770 echocardiograph with 704B probe, Visualsonics, Toronto, CA. USA). The heart was imaged in the two-dimensional mode (M-mode) in the parasternal long axis views. The measurements of intra-ventricular septal (IVS) thickness, left ventricular posterior wall thickness (LWPW), and left ventricular internal diameters were made in systole and diastole. Left ventricular percent fractional shortening, ejection fraction, chamber volume and mass were calculated using methods as previously described. [4]

In Vivo Assessment of Mouse Left Ventricular Contractile Function In vivo LV function was assessed by PV catheter. Briefly, mice were anesthetized with pentobarbital (50 mg/kg), incubated and ventilated with a custom-designed constant-pressure ventilator with 100% oxygen at 120 breaths/min and a tidal volume of 200 μl. The LV apex was exposed and 1.4 F PV catheter (SPR 839; Millar Instruments, Houston,TX) was advanced through apex along the longitudinal axis. PV data were measured at steady state and during transient reduction in venous return by occluding the inferior vena cava [5]. For carotid approach, GRK2 transgenic mice were anesthetized with a mixture of ketamine (100 mg/kg) and xylazine (2.5 mg/kg) as previously described[6, 7]. A cervical middle line skin incision was made; right common carotid artery was isolated or chest opened and cannulated with 1.4 F PV catheter through right carotid artery. LV pressures, LV end-diastolic pressure (LVEDP) and heart rate (HR) were measured by this catheter advanced into the LV cavity, and data was recorded and analyzed on a PowerLab System (AD Instruments Pty Ltd., Mountain View, CA) to obtain dP/dt max as a measure of global systolic function and dP/dt min as a measure of global diastolic function. The right jugular vein was canulated with PE10 tubing and after recording the baseline hemodynamic data, the βAR agonist, isoproterenol (ISO, 0.5 ~ 7.5 ng) was administered through i.v. delivery line.

Radioligand Binding Heart tissues were harvested in lysis buffer (5 mM Tris-HCl, pH 7.4, with 5 mM EGTA) and homogenized with 15 strokes on ice. Samples were centrifuged at 30,000g for 15 min to pellet membranes. The membrane proteins were then resuspended in binding buffer (75 mM Tris-HCl, pH 7.4, 12.5 mM $MgCl_2$, 2 mM EDTA) and stored in aliquots at -80°C. Binding assays were performed on 5-25 μg of membrane proteins using saturating amounts of the βAR-specific ligand [^{125}I]-iodocyanopindolol ([^{125}I]-CYP), as described previously [8]. Saturation experiments were performed with [^{125}I]-CYP concentrations ranging from 1 to 300 pM in the absence or presence of 1 μM propranolol to determine total and non-specific binding, respectively. The competition binding was performed with ICI118551 in the presence of 50 pM of ^{125}I-CYP. Radioligand binding reactions were terminated by dilution and rapid filtration over glass fiber filters. All assays were performed in duplicate, and receptor density was normalized to milligrams of membrane proteins.

Western Blot Analysis For western blot analysis, cell extracts were electrophoresed on a 4-12% NuPAGE gel (Invitrogen) and transferred to the polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA). The membrane was blocked with 5% non-fat dry milk in Tris-buffered saline-0.1% Tween-20 (TBS-T) and incubated overnight with an anti-GRK2(Santa Cruz, SC-562), or anti-phospho-ERK1/2 (New England Biolabs), or anti-actin (Santa Cruz, SC-1616) Anti-G_{i-3} (Santa Cruz, SC-262), Anti-β_2-AR (Santa Cruz, SC-569). The antibodies for phosphorylated β_2AR were raised against the peptides CDRTGHGLRRSpSKF-NH2 for the anti-pSer262 PKA site (clone 2G3) and CKAYGNGYpSpSNGN-NH2 for the anti-pS (Ser355, 356) (clone 5C3). They were a gift from Dr. Richard B. Clark. Membranes were washed and then incubated with secondary antibody conjugated with horseradish peroxidase in 5% non-fat milk in TBS-T buffer for 1-2 h. Detection was carried out using a chemiluminescence detection kit (Cell Signaling Technology) and quantified by scanning laser densitometry.

Adult Mouse Cardiac Myocytes Culture, Adenoviral Gene Transfer, and Survival Assay Single mouse cardiac myocytes were isolated from 2~3-month-old C57/B6 mice with an enzymatic technique [9]. Cells were then cultured and infected with adenoviral vectors, as described previously [9]. Before culture, myocytes were washed three times with minimal essential medium (MEM) containing 1.2 mM Ca^{2+}, 2.5% fetal bovine serum (FBS), and 1% penicillin-streptomycin and then plated with the same medium in the culture dishes pre-coated with 10 μg/ml mouse laminin. Adenovirus-mediated gene transfer was implemented by adding a minimal volume of the FBS-free MEM containing an appropriate titer of gene-carrying adenovirus. The full volume of FBS-free MEM was supplied after culture for another 1 to 2 h. All experiments were performed after 24 h of adenoviral infection. The cultured myocytes was stained with 1 μg/ml of Propidium iodide (Calbiochem, San Diego, CA)[10].

Cardiac Myocytes Contraction Measurements Cells were placed on the stage of an inverted microscope (Zeiss, model IM-35), perfused with a Hepes-buffered solution containing (in mM) 137 NaCl; 5.4 KCl; 1.2 $MgCl_2$; 1 NaH_2PO_4; 1 $CaCl_2$; 20 Glucose and 20 Hepes (pH 7.4), and electrically stimulated at 0.5 Hz at $23^{o}C$. Cell length was monitored by an optical edge-tracking method using a photodiode array (Model 1024 SAQ, Reticon) with a 3 ms time resolution. Cell contraction was measured by the percent shortening of cell length following electrical stimulation[11]. To inhibit G_i signaling, subset of freshly isolated or cultured myocytes were incubated with PTX (1.5 μg/ml, at 37^0C for at least 3 hr), prior to contraction measurements. PTX-treated cells were compared with myocytes which had been kept at $37^{o}C$ in the absence of PTX for an equal period of time.

Histological analysis Heart specimens were fixed with 10% neutral buffered formalin, embedded in paraffin, and sectioned at 5-μm thickness. Sections were stained with H&E and Elastic Picro Sirius Red (E.P.S.R). We determined cardiomyocyte diameter and interstitial collagen fraction using computer-assisted image analysis (Image J).

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) Staining

CardioTACS in Situ apoptosis detection kit (R and D Systems Inc. no. TA5353; Minneapolis, MN) was used to detect DNA fragmentation in tissue sections. For each sample, two slides were stained. From each slide, 16 × 10 fields of view were digitized for analysis. TUNEL positive nuclei and total nuclei were then counted for each image, tallied for each slide, and averaged for each sample.

cAMP Measurement Intracellular cAMP levels were assayed by radioimmunoassay. The cardiomyocytes from NTG, WT-, PKA-, GRK- TG β_2AR mice were isolated and plated in laminin-coated 6-weel plates. Four hours after cell were seeded, cells were stimulated with 10 uM of isoproterenol at designed time point. Five minutes before stopping reaction at designed time points, cells were treated with 200 uM of IBAX (3-isobutyl-1-methylxanthine) to accumulate cAMP. The cell lysates were committed to cAMP assay according to the Assay kit (Parameter TM, R&D Systems, KGE002B) with a duplicate in each experiment. Protein content was measured using the Piece BCA (Thermo Scientific 23228).

Statistical Analysis Data were expressed as mean ± s.e.m. Differences between multiple groups were compared by analysis of variance (ANOVA) followed by a Bonferroni's multiple comparisons test. Two-group analysis was performed by t-test. Serial studies were tested by repeated measures ANOVA. Survival curves were analyzed by Kaplan-Meier Survival Analysis.

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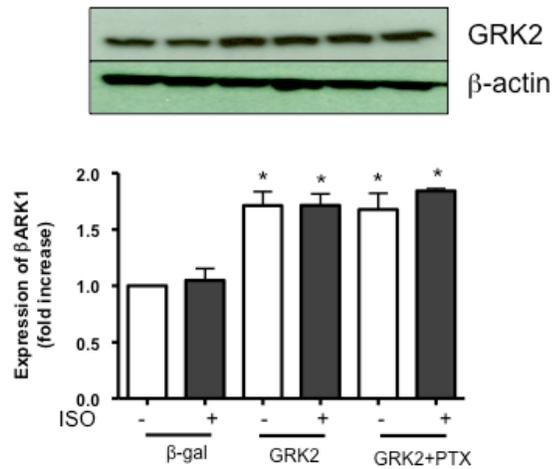
Online Figure I. ISO or PTX incubation did not change the GRK2 expression in NTG mice cardiomyocytes cultured for 24 hours. After cells were plated, cells were further cultured in the presence of ISO (1 nM) with or without PTX (0.5 µg/ml) for 24 hours. At this concentration of ISO or PTX, no cardiomyocytes was died compared to no any treatment cells, as evidenced by PI staining. n=3, *p<0.01 *v.s.* β-gal group.

Online Figure II
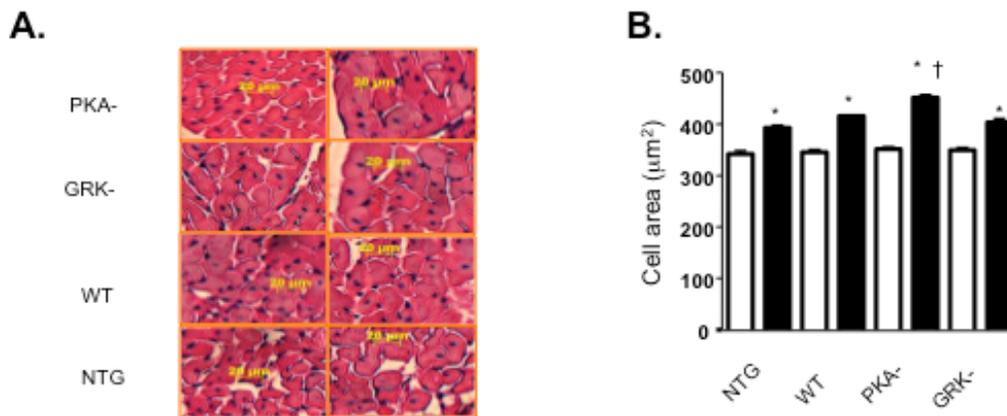
Online Figures II: Overexpression of β_2AR mutant lacking PKA phosophorylation sites exaggerated cardiomyocyte hypertrophy in response to pressure overload. (A) Representative photomicrographs illustrate ventricular myocyte cross sections in NTG, WT TG, PKA- TG and GRK2-TG mice. (B) Average data on ventricular myocytes cross sections (369 and 412 cells from 4 hearts for NTG Sham and TAC, respectively; 425 and 434 cells from 4 hearts for WT TG Sham and TAC, respectively; 484 and 450 cells from 4 hearts for GRK2- TG Sham and TAC, respectively; 351 and 451 cells from 5 hearts for PKA- TG Sham and TAC, respectively; *p<0.001 *v.s.* respective control; †p<0.001 *v.s.* other three groups with TAC).
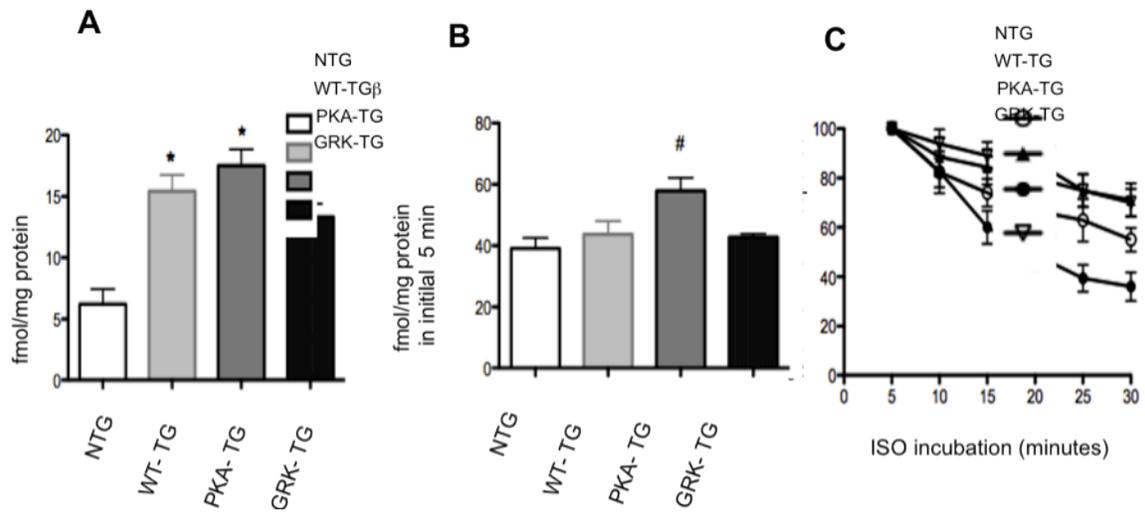
Online Figure III. The cardiac myocytes from PKA- TG mice was facilitated to decay of cAMP accumulation through PTX sensitive pathway. The basal cAMP (A), maximum response (initial 5 min) (B), and decay of cAMP accumulation (C) in the cardiac myoycts from NTG, WT-, PKA-, GRK-TG mice. The cardiomyocytes from NTG, WT-, PKA-, GRK- TG mice were isolated and plated in laminin-coated 6-weel plates. Four hours after cell were plated, cells were stimulated with 10 μM of isoproterenol (ISO) at designed time. 5 minutes before stopping reaction at designed time points, cells were treated with 200 μM of IBMX (3-isobutyl-1-methylxanthine) to accumulate cAMP. The cell lysates were committed to cAMP assay according to the assay kit. *P<0.05 *v.s.* NTG , #P<0.05 *v.s.* NTG, GRK-, WT-TG, n=3-4.

Online Figure IV
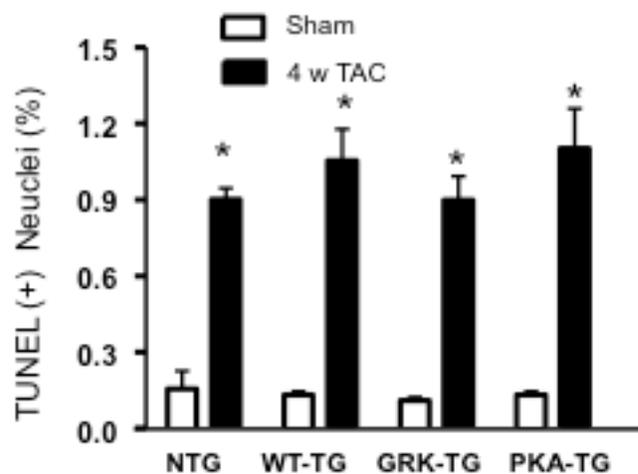
Online Figure IV. Pressure over-load induced the myocardium apoptosis in NTG, WT-, PKA-, GRK-β_2AR TG mice, but no difference between different transgenic β_2AR mice. Heart specimens were fixed with 10% neutral buffered formalin, embedded in paraffin, and sectioned at 5-μm thickness. For each sample, two slides were stained. From each slide, 10 fields of view (200X) were digitized for analysis. TUNEL positive nuclei and total nuclei were then counted for each image, tallied for each slide, and averaged for each sample. *P<0.01 vs sell sham group, n=3 hearts.
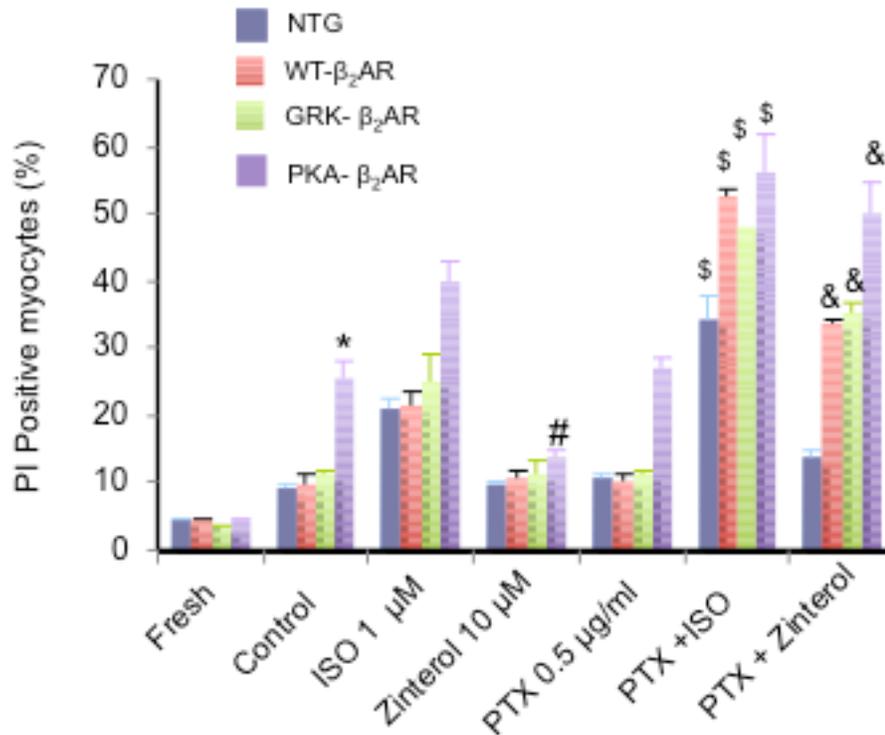
Online Figure V. Cardiomyoctes with high expression of phosphorylation of β_2AR in GRK sites (PKA-β_2AR) were vulnerable to βAR agonist stimulation when inhibition of G_i-signaling with PTX. The cardiomyocytes from NTG, WT-, PKA-, GRK-TG β_2AR mice were cultured for 24 h with or without isoproterenol (1 μM) or zinterol (10 μM) in the absence or presence PTX (0.5 μg/ml). The cells were stained with Propidium iodide (1 μg/ml). The death cell was seen as nuclear positive staining. N=3-4, * $P<0.01$ vs NTG, WT, GRK-TG; # $P<0.05$ *v.s.* ISO PKA-TG and control PKA-TG group; $ $P<0.05$ *v.s.* ISO alone self-strain; & $P<0.05$ *v.s.* Zinterol alone.

Table I. Characteristics of β_2AR in hearts from NTG, WT-TG, PKA-TG, and GRK-TG mice

Groups	β-AR (fmol/mg protein)	Kd (pM)	β_2AR %	β_2-AR (fmol/mg protein)	β_1-AR (fmol/mg/protein)
NTG Sham (4)	28.6±3.4	64.0±4.9	30.5±2.5	8.7±1.3	20.8±2.7
NTG TAC (3)	20.5±2.3	53.6±16.2	28.3±3.6	5.8±2.3	14.7±2.4*
WT-TG Sham (3)	856±45	65.5±5.4			
WT-TG TAC (3)	764±48	63.3±6.8			
GRK-TG SHAM (3)	850±23	82.6±13.3			
GRK-TG TAC (3)	812±25	76.4±12.4			
PKA-TG Sham (3)	828±33	73.6±3.6			
PKA-TG TAC (3)	750±48	70.4±7.9			

N=3-4, mean ± SE, *P<0.05 vs NTG sham

Table II. Affinity of β_2AR for ICI 118551

Groups	ICI118551 kH (nM)	kL (nM)	High, %	nHill
NTG Sham (4)	1.23±0.54	1364±353	30.5±2.5	0.456±0.023*
NTG TAC (3)	1.14±0.32	1253±328	28.3±3.6	0.435±0.021*
WT-TG Sham (3)	1.45±0.54	N/A		0.765±0.123
WT-TG TAC (3)	1.38±0.44	N/A		0.758±0.249
GRK-TG SHAM (3)	1.25±0.54	N/A		0.835±0.215
GRK- TAC (3)	1.13±0.32	N/A		0.923±0.325
PKA-TG Sham (3)	1.36±0.18	N/A		0.759±0.265
PKA-TAC (3)	1.39±0.23	N/A		0.843±0.313

N=3-4, mean ± SE, *P<0.05 vs unity