Supporting Information

Gesmundo et al. 10.1073/pnas.1712612114

SI Methods

Reagents. M1018 medium, phenylephrine, FSK, SYBR Green dye, anti–α-actinin antibody, ESI-09, metoprolol, and cell culture reagents were from Sigma-Aldrich. KT5720 was from Biomol Research Laboratory Inc. (DBA), U73122 and 6-Bnz-cAMP were from Tocris (Space Import Export). P-PKCε (Ser729), Epac1, and SERCA2a antibodies were from Cell Signaling Technology (Euroclone), GAPDH antibody was from Millipore. Calcineurin A antibody was from Abcam. P-phospholamban (Ser16) and P-phospholamban (Thr17), PLCβ, total antibodies, and actin were from Santa Cruz Biotechnology (DBA). RT-PCR and real-time PCR reagents were from Life Technologies. Primers were from IDT (TemaRicerca).

Cell Lines. The embryonic rat heart-derived cell line H9c2 was purchased from American Type Culture Collection and cultured as described previously (7). Cells were maintained in 100-mm dishes at 37 °C with 5% CO_2 in DMEM with 10% FBS, 4 mM glutamine, 1% penicillin-streptomycin, and grown to subconfluence before experiments.

Isolation of ARVMs. ARVMs were obtained from young adult (1–3 mo) rats by enzymatic dissociation and cultured as described previously (7). All procedures were approved by the Animal Care and Use Committee of the University of Turin, in accordance with the European Directive 2010/63/EU. All solutions used for dissociation contained 10 mM butanedionemonoxime (BDM) to inhibit excitation–contraction coupling. Explanted hearts were cannulated via the aorta and perfused at constant flow rate (5 mL/min) with a peristaltic pump for 5 min with 0 Ca^{2+} Tyrode solution. The latter and the following operations were carried out under a laminar flow hood. The heart was then perfused for 2 min with 0 Ca^{2+} Tyrode collagenase and finally with the same Tyrode-collagenase plus 50 μM Ca^{2+}, to facilitate cellular dissociation. The heart was detached from the cannula and the ventricles cut away and minced in small fragments. The fragments were collected in Tyrode-collagenase plus 50 μM Ca^{2+} solution and gently mixed. After 10 min, the medium was replaced with Tyrode 50 μM Ca^{2+} and mixed again with ventricular fragments for 10 min. The supernatant (more than 50% rod-shaped cells) was collected, filtered, and exposed to growing concentrations of $CaCl_2$ (from 50 μM to 700 μM) before the cells were used. For experiments, about 10,000 CMs were plated, respectively, on laminin-treated dishes and incubated in M1018 medium with 10 mM BDM Sigma-Aldrich, 100 U/mL penicillin, 100 μg/mL streptomycin, and insulin, transferrin, selenium (1:1,000; Sigma). CMs were then placed in a 37 °C, 5% CO_2 incubator, until adhesion occurred, for at least 2 h.

Staining for α-Actinin and Assessment of Cell Surface Area. H9c2 cells and ARVMs were grown into glass coverslips in 24-well plates. After 48 h, cells were serum-starved for 24 h and incubated with the different stimuli. Cells were permeabilized with 0.1% Triton X-100 after fixation in paraformaldehyde, blocked in 1% newborn goat serum for 1 h at room temperature, and stained overnight at 4 °C with α-actinin antibody (1:800). The cells were then incubated for 1 h at room temperature with Alexa Fluor-546 antibody (1:450; Life Technologies). For H9c2 cells, nuclei were stained with Hoechst 33258 (1:1,000) for 10 min at 4 °C. H9c2 cells images were taken using a Leica DM200 fluorescent microscope and a Leica DFC340 FX camera (magnification, 100×) and analysis performed with a Leica Suite image analysis software. For ARVMs, images were captured with an Olympus Fluoview 200 confocal head with an Ar/Kr laser (488 and 568 nm; magnification, 60×). Area calculation was performed using a computerized morphometric system (Image J software, https://imagej.nih.gov/ij/), counting 50 cells from at least 20 randomly chosen fields for each condition.

RT-PCR and Real-Time PCR. Extraction of total RNA from H9c2 cells, ARVMs, and human iPSC-CMs, and reverse transcription were performed as described previously (7). Briefly, the cDNA was amplified with the AmpliTaq Gold Polymerase in a GeneAmp PCR System (Perkin-Elmer). For GHRH-R and GHRH, a second PCR (40 cycles) was performed on the primary PCR products. The annealing temperature was 64 °C and 65 °C, for the first and the second PCR, respectively, for GHRH-R; 56 °C for both GHRH PCRs, and 62 °C for SV1. The primers, designed using Primer3, spanned the first amplification product (Table S1). β-Actin mRNA served as internal control; the negative control consisted of no RNA. The LNCaP human prostate cancer cell line (American Type Culture Collection) was used as positive control. The PCR products were separated by gel electrophoresis and visualized by ethidium bromide staining. For real-time PCR, cDNAs were treated with DNA-free DNase (Life Technologies) and reaction performed with the SYBR Select Master Mix (ThermoFischer Scientific) using the ABI-Prism 7300 (Applied Biosystems) or ViiA 7 Real Time (Thermo Fisher Scientific). Disruption of isolated mice CMs was performed for 3 min at 30 Hz, using Tissue Lyser (Qiagen), and total RNA extracted using RNeasy Mini Kit (Qiagen); cDNA was synthesized using SuperScript Vilo cDNA Synthesis Kit (Life Technologies). The primer sequences are reported in Table S2. 18s rRNA was used as endogenous control. Relative quantification was performed using the comparative Ct (2–ΔΔCt) method.

PKA Activity Assay. Cells were seeded in 100-mm dishes at a concentration of 5×10^5 cells. After 48 h, cells were serum-starved for 24 h and incubated with the different stimuli for 15 min. PKA activity was measured from cell lysates using the PKA Kinase Activity Assay kit (Abcam), according to the manufacturer's instructions. Relative kinase activity (Abs_{450}) was then calculated using the following formula: Av $Abs_{(sample)}$ − Av $Abs_{(blank)}$, where Av $Abs_{(sample)}$ is the average absorbance of the sample, and Av $Abs_{(blank)}$ is the average absorbance of the blank. The blank consisted of the provided kinase assay dilution buffer (no active kinase).

Western Blot Analysis. Western blotting was performed as described previously (10). Proteins (50 or 60 μg) were resolved in 10% or 13% SDS/PAGE and transferred to a nitrocellulose membrane. Membranes were incubated with the specific antibody (calcineurin A, Epac1, P-PKCε, P-phospholamban, PLCβ1, SERCA2a) (dilution 1:1,000). Blots were reprobed with total antibodies or actin for normalization. Immunoreactive proteins were visualized using horseradish peroxidase-conjugated goat anti-mouse or goat anti-rabbit (1:5,000) by enhanced chemiluminescence substrate using ChemiDoc XRS (Bio-Rad), densitometric analysis performed with Quantity One software (Bio-Rad). In mouse experiments, hearts were lysed in Tris-buffered saline with 1% Triton X-100, containing Roche complete protease inhibitor mixture, 10 mM NaF, 1 mM PMSF, and 1 mM Na_3VO_4. Protein extracts were prepared as previously described

(37). Protein quantifications were performed with Quantity One software (Bio-Rad).

Generation of Human iPSC-CMs. iPSC-CMs were differentiated from human iPSCs, previously generated from skin fibroblasts of healthy individuals, as described previously (36). Cardiac induction was performed using a chemically defined and xeno-free protocol adapted from a method by Lian et al. (41) and that relies on modulation of the Wnt pathway (42). Induction of hypertrophy was carried out using 100 μM PE on CM cultures at day 20 after the start of spontaneous contraction. GHRH was administered at the same time at the final concentration of 0.5 μM, alone or with PE.

Animals. All procedures on mice were performed according to institutional guidelines in compliance with national (D.L. N.26, 04/03/2014) and international law and policies (new directive 2010/63/EU). The protocol was approved by the Italian Ministry of Health. TAC or sham surgery was performed in 8-wk-old C57BL/6J male mice as described previously (38). Mice were anesthetized by intraperitoneal injection with ketamine (100 mg/kg) and xylazine (10 mg/kg). The pressure load was verified through measurement of the pressure gradient across the aortic constriction with echocardiography (Vevo 2100; VisualSonics). Fourteen-days postoperation, mice underwent echocardiography analysis to evaluate heart function and pressure gradient. Only mice with a gradient between 60 and 90 mmHg were included in the experiment. Fourteen days after operation, mice were subcutaneously injected with MR-409 (500 μg/kg/d for 14 d) or vehicle, and killed 14 d after treatment. The hearts were excised and CMs isolated by enzymatic perfusion of the left ventricle with Liberaze TM (Roche), using a Langerdorff apparatus, as previously described (38).

Isolation of Adult Mice CMs. CMs were isolated from adult male mice using standard enzymatic techniques as previously described (38). Freshly isolated CMs were plated at a density of $0.5–1 \times 10^4/cm^2$ in 22-mm coverslips precoated with 20 μg/mL laminin (Life Technologies) in HBSS supplemented with 0.01 mM $CaCl_2$. Coverslips were mounted on a temperature-controlled perfusion chamber (University of Berne, Switzerland) and slowly superfused with HBSS (Sigma) supplemented with 10 mM Hepes, pH 7.4, to reach the final Ca^{2+} concentration of 1.1 mM.

Cell Contractility. CMs were freshly isolated from TAC mice (control) and from TAC mice treated with MR-409. Cells were loaded with 1 μM Fura-2 acetoxymethyl (Thermo Fisher Scientific), field stimulated at 0.5, 1.0, 2, and 3 Hz and recorded using an IonOptix System, as previously described (40). The cells were stimulated with depolarized square pulses at 0.5, 1, 2, and 3 Hz and the following parameters were analyzed during contraction/relaxation periods: cell shortening, time-to-baseline calculated at 90%, and baseline percentage to peak for the intracellular calcium transient.

GH and IGF-I Analysis. Total blood from the facial vein of TAC mice was collected at the time of killing. Serum, obtained by centrifugation of total blood at $450 \times g$ at 4 °C for 15 min, was stored at −80 °C until analysis. GH and IGF-I were measured using mouse GH ELISA kit (Cusabio, Space Import Export) and mouse IGF-I ELISA Kit (Abcam) respectively, following the manufacturers' instructions.

Histochemistry and Cell Size Analysis in CMs Isolated from TAC Hearts. Mouse hearts were harvested and fixed overnight in formalin, followed by paraffin embedding and sectioning. Histochemical analysis was performed on 3-μm-thick paraffin sections and analyzed by staining with H&E using an Olympus VS120 Virtual Slide Microscope. Isolated adult CMs were acquired with a microscope and converted to greyscale. Cells size measurements were performed using Image J software, according to the user's guide.

***Z*-Groove Ratio Analysis.** The *z*-groove ratio was calculated from topographical images (10 × 10 μm) of cells obtained by HPICM with continuous feedback, as previously described (27). The scanned topographical images were used to quantify disruption of surface structural regularity in TAC mice subjected to vehicle (DMSO) or daily injection of MR-409 for 14 d. Briefly, a piezo-controller (ICnano Scanner Controller; Ionscope Ltd) controlled the three-axis (*xyz*) piezo translation stage (Physik 140 Instruments) custom-assembled with 100-μm closed-loop travel range in *x* and *y*, and 50-μm in the *z* direction. The piezo-stage was driven by a high-voltage amplifier (Physik Instruments) connected to a scanner controller (IC-Nano). The pipette electrode head-stage was connected to a Multiclamp 700B (Molecular Devices). The scan head was assembled onto an electrical micromanipulator (Scientifica) based on a motorized platform (Scientifica). Preparations were imaged with a Nikon TE-i inverted microscope (Nikon Corporation). Nanopipettes (∼80 MΩ tip-resistance) were pulled from borosilicate glass with O.D. 1.0 mm and I.D. 0.58 mm (Intracell) using a laser puller P-2000 (Sutter Inc.). Nanopipettes were filled with HBSS without calcium (Sigma Aldrich) containing 10 mM Hepes (Lonza), 0.1 mM Ca^{2+}, pH 7.4. Low extracellular calcium concentration is imperative for avoiding undesirable contraction during scan acquisition. Surface topographical images were acquired by HPICM at 25 °C in the same HBSS low-calcium solution. Acquisitions were obtained with customized software, and analysis performed with Prism GraphPad software 6.0.

Statistical Analysis. Results are presented as mean ± SEM. Significance was calculated by two-tailed Student's *t* test or two-way ANOVA followed by Bonferroni's multiple comparison test for post hoc analysis. Analysis was performed using GraphPad Prism 6.0. Significance was established for $P < 0.05$.

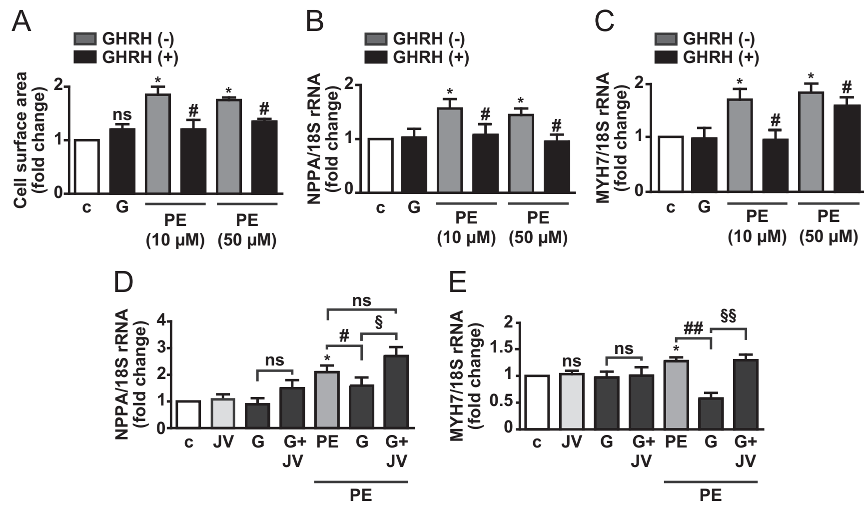

Fig. S1. Antihypertrophic effects of GHRH in H9c2 cardiac cells. The cells were serum-starved for 24 h, then incubated with GHRH (0.5 μM) or PE (10 and 50 μM) for 24 h, or preincubated with GHRH for 40 min, then treated with PE for further 24 h. (*A*) Cell surface area measured in cells stained with α-actinin. The relative area, normalized to the control, was analyzed in 50 cells for each condition from at least 20 randomly chosen fields. Results, expressed as fold-change over control, are mean ± SEM $*P < 0.05$ vs. c; $^{\#}P < 0.05$ vs. PE; ns, not significant; $n = 3$. (*B* and *C*) Real-time PCR analysis of *NPPA* and *MYH7* mRNA normalized to 18S rRNA and expressed as fold-change vs. control (c). Results are shown as mean ± SEM $*P < 0.05$ vs. c; $^{\#}P < 0.05$ vs. PE alone, at each concentration; ns, not significant; $n = 3$. (*D* and *E*) *NPPA* and *MYH7* mRNA analyzed by real-time PCR in cells untreated (control, c) or treated for 20 min with JV-1-36 (50 nM), then for 40 min with GHRH (G, 0.5 μM) and for 24 h with PE (10 μM). $*P < 0.05$ vs. c; $^{\#}P < 0.05$; $^{\#\#}P < 0.01$; $^{\S\S}P < 0.01$.

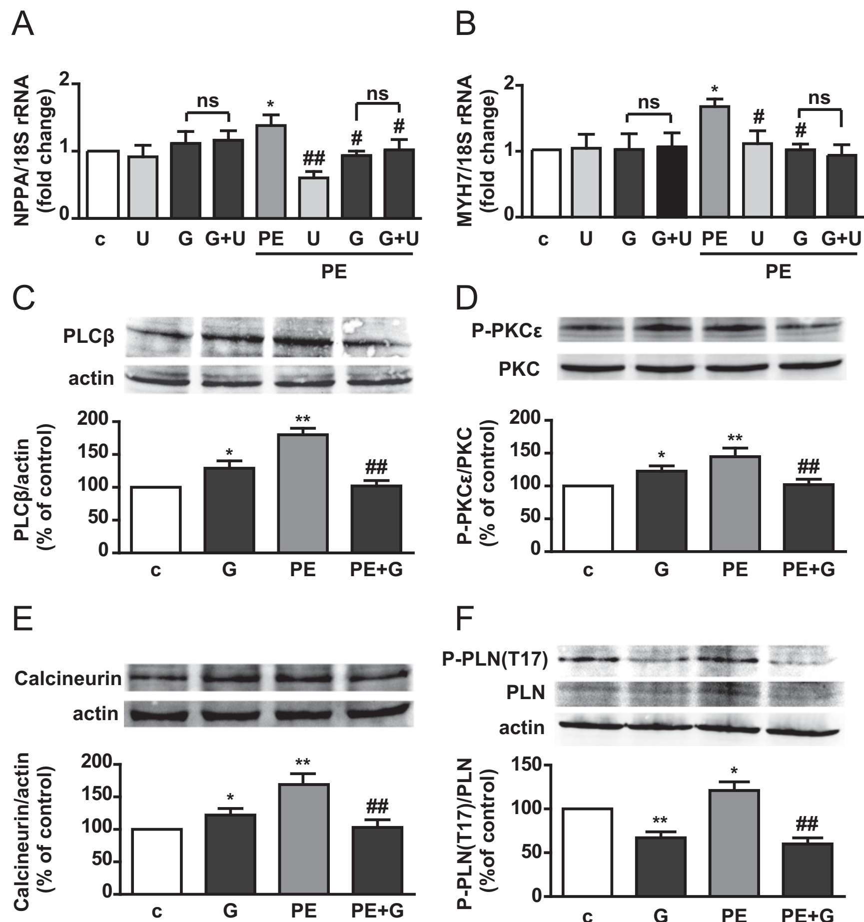

Fig. S2. Inhibitory effect of GHRH on PE-induced activation of Gq/PLCβ signaling. (*A* and *B*) *NPPA* and *MYH7* mRNA assessed by real-time PCR in H9c2 cells treated for 20 min with or without 0.5 μM U-73122 (U), then for 40 min with 0.5 μM GHRH and for further 24 h with 10 μM PE. Results, normalized to 18S rRNA, are expressed as mean ± SEM $*P < 0.05$ vs. c; $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ vs. PE; ns, not significant; $n = 3$. (*C*–*E*) Representative Western blot for PLCβ expression (*C*), PKCε phosphorylation (*D*), and calcineurin expression (*E*) in cells treated for 24 h with the indicated stimuli (*Upper*). Proteins were reprobed with antibody to actin (*C* and *E*) or to total PKCε (*D*) (*Lower*). Graphs show the densitometric analysis of normal or phosphorylated proteins, normalized to actin or their respective total proteins, and reported as percent of basal. Results are presented as mean ± SEM $*P < 0.05$ and $**P < 0.01$ vs. c; $^{\#\#}P < 0.01$ vs. PE; $n = 3$. (*F*) PLN phosphorylation at Thr17 assessed by Western blot in cells stimulated for 30 min with 0.5 μM GHRH, 10 μM PE, or a combination of both (*Top*). Blots were reprobed with antibody to total PLN (*Middle*) and to actin (*Bottom*). Phosphorylated PLN was normalized to total PLN and reported as percent of control. Results are mean ± SEM $*P < 0.05$ and $**P < 0.01$ vs. c; $^{\#\#}P < 0.01$ vs. PE; $n = 3$.

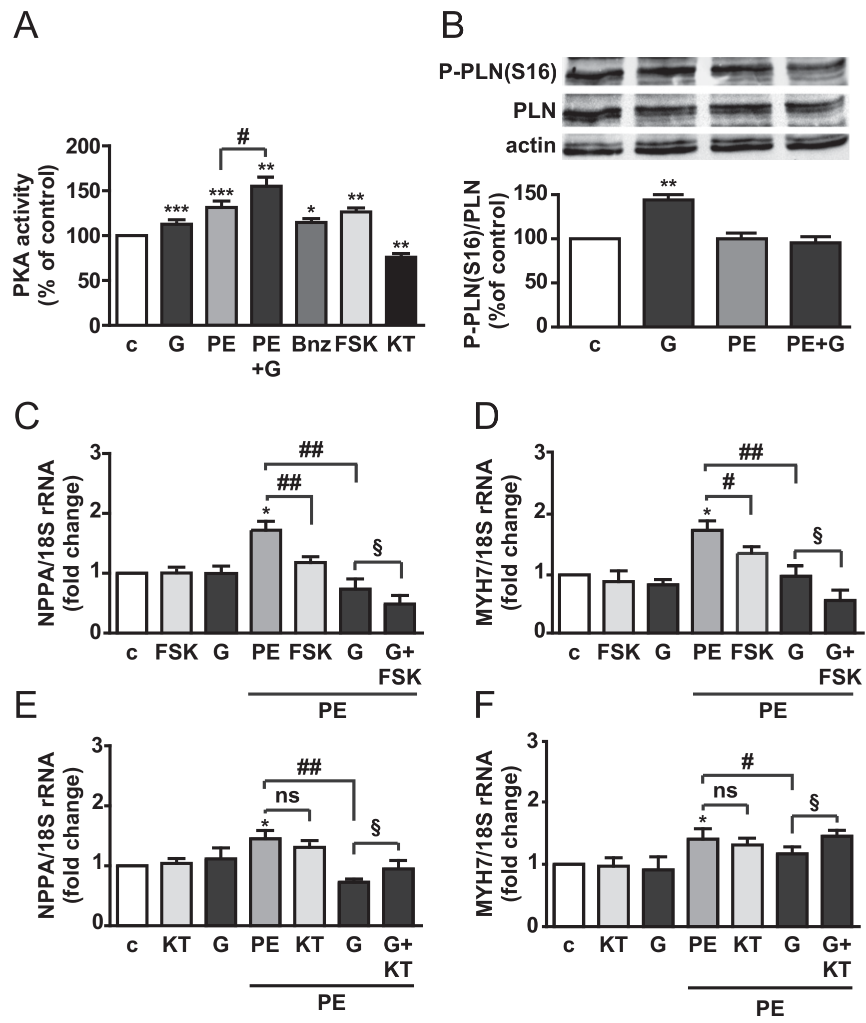

Fig. S3. AC/cAMP/PKA-dependent antihypertrophic effects of GHRH. (*A*) PKA activity in H9c2 cells, untreated (control, c) or treated for 15 min with GHRH (G, 0.5 μM) or PE (10 μM), alone or in combination, and with 6-Bnz-cAMP (Bnz, 300 μM), KT5720 (KT, 0.1 μM), or FSK (10 μM). Results are mean ± SEM $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$ vs. c; $^{\#}P < 0.05$; $n = 3$. (*B*) Western blot analysis for PLN phosphorylation at Ser16 in H9c2 cells stimulated for 30 min with 0.5 μM GHRH, 10 μM PE or a combination of both (*Top*). Blots were reprobed with antibody to total PLN (*Middle*) and to actin (*Bottom*). Phosphorylated PLN was normalized to total PLN and reported as percent of control. Results are shown as mean ± SEM $**P < 0.01$ vs. c; $n = 3$. (*C–F*) *NPPA* and *MYH7* mRNA assessed by real-time PCR in cells untreated (control, c) or treated for 20 min with FSK (10 μM) (*C* and *D*) or KT5720 (0.1 μM) (*E* and *F*), then for 40 min with GHRH (G, 0.5 μM), and an additional 24 h with PE (10 μM). Results, normalized to 18S rRNA, are expressed as fold-change over control and are mean ± SEM $*P < 0.05$ vs. c; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$; $^{§}P < 0.05$; ns, not significant; $n = 3$.

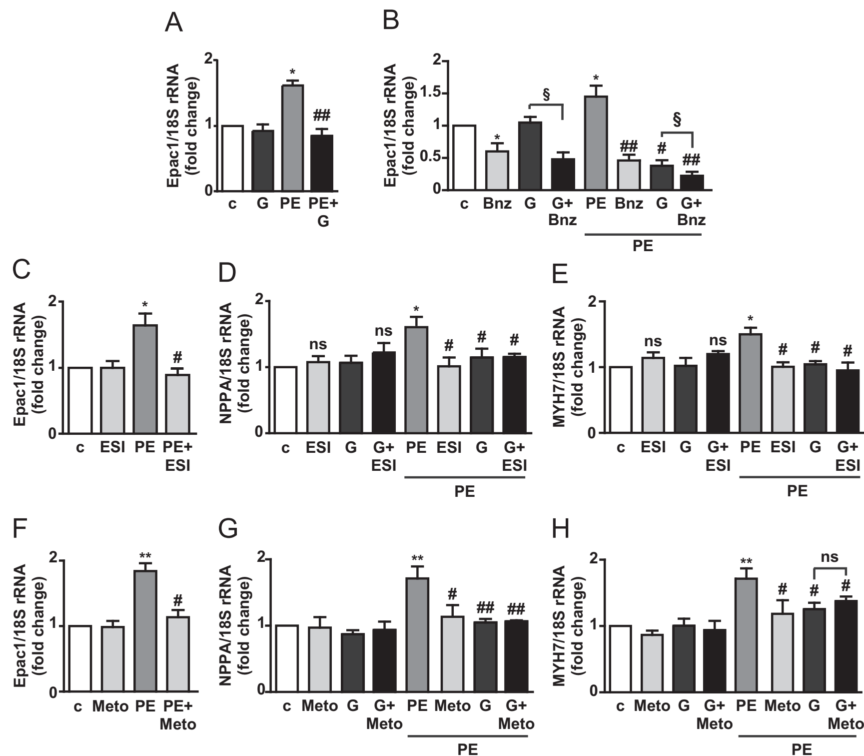

Fig. S4. GHRH inhibits PE-induced increase in Epac1. (*A*) *Epac1* mRNA assessed by real-time PCR and normalized to 18S rRNA in H9c2 cells untreated (control, c) or treated for 40 min with GHRH (G, 0.5 μM) and for an additional 24 h with PE (10 μM). Results, expressed as fold-change of control, are presented as mean ± SEM $^{*}P < 0.05$ vs. c; $^{\#\#}P < 0.01$ vs. PE; $n = 3$. (*B*) *Epac1* mRNA assessed by real-time PCR (normalized to 18S rRNA) in cells pretreated for 30 min with 300 μM 6-Bnz-cAMP (Bnz), then for 40 min with 0.5 μM GHRH and for 24 h with 10 μM PE. Results are mean ± SEM $^{*}P < 0.05$ vs. c; $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ vs. PE; $^{\S}P < 0.05$; $n = 3$. (*C*–*H*) Real-time PCR for *Epac1*, *NPPA*, and *MYH7* in cells untreated or pretreated for 30 min with ESI-09 (10 μM) (*C*–*E*), or metoprolol (Meto, 0.1 μM) (*F*–*H*), then for 40 min with GHRH (G, 0.5 μM) and for an additional 24 h with PE (10 μM). Results, normalized to 18S rRNA, are expressed as fold-change over control and are mean ± SEM $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. c; $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ vs. PE; $n = 3$.

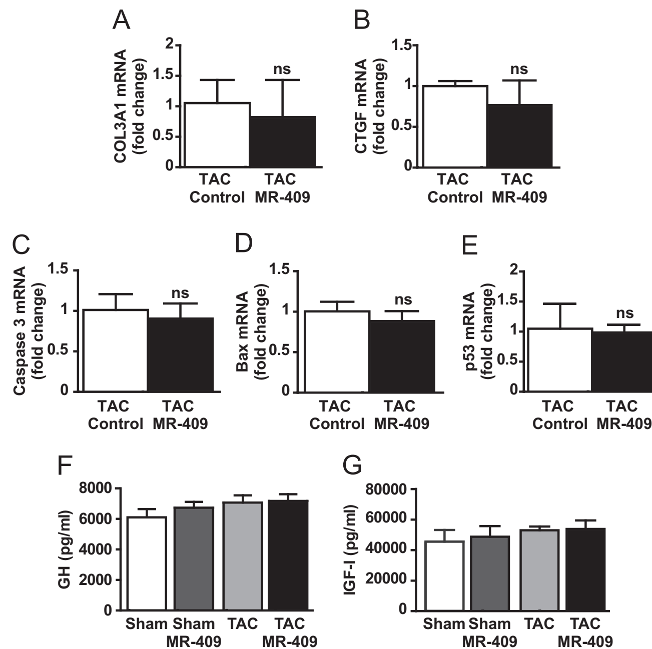

Fig. S5. Expression of genes for fibrosis and apoptosis in CMs and serum levels of GH and IGF-I in TAC mice treated with MR-409. CMs were isolated from mice subjected to TAC for 4 wk (TAC control) and from TAC mice treated for 2 wk with MR-409 (TAC MR-409). mRNA levels for collagen type III α 1 chain (COL3A1) (*A*), connective tissue growth factor (CTGF) (*B*), caspase 3 (*C*), Bax (*D*), and p53 (*E*) assessed by real-time PCR and expressed as fold-change of TAC control. Results are mean ± SEM from three independent experiments (ns, not significant). (*F* and *G*) Serum levels of GH and IGF-I determined by ELISA from the facial vein of mice subjected to Sham or TAC for 4 wk, and either untreated or treated for 2 wk with MR-409. Results are mean ± SEM. All results are not significant for Sham MR-409 vs. Sham, for TAC vs. Sham and for TAC MR-409 vs. TAC ($n = 5$, each performed in triplicate).

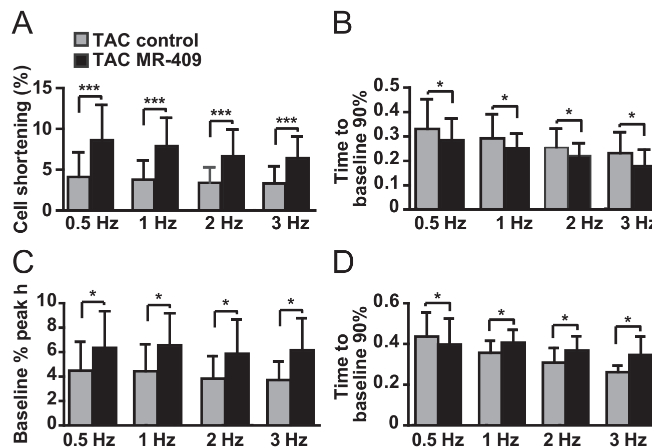

Fig. S6. Contractility and calcium transients in CMs from TAC mice treated with MR-409. (*A*) Cell shortening (%) at different stimulation frequencies, $n = 43$. (*B*) Same as *A* for the time-to-baseline calculated at 90%, $n = 43$. (*C*) Baseline percentage to peak for the intracellular calcium transient, $n = 21$. (*D*) Same as *C* for the time-to-baseline (calculated at 90%), $n = 15$. Results are mean ± SEM $^*P < 0.05$; $^{***}P < 0.0001$.

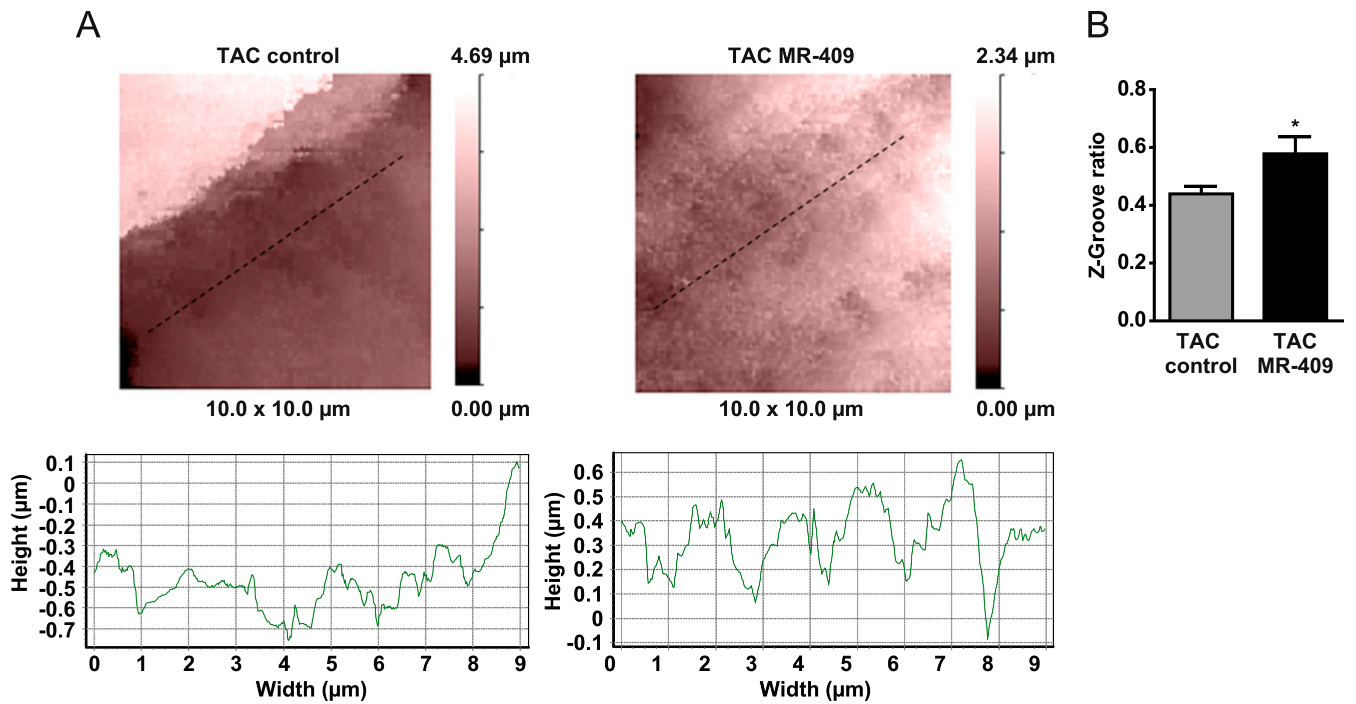

Fig. S7. Surface morphology in CMs isolated from TAC mice treated with MR-409. (*A*, *Upper*) Representative SICM image of a ventricular CM isolated from untreated TAC mouse (*Left*, TAC control) and from a TAC mouse treated with MR-409 (*Right*, TAC MR-409). (*Lower*) Surface topographical profiles of crest and *z*-grooved related to a dashed blacked line drawn on the corresponding *Upper* images. (*B*) The *z*-groove ratio calculated from TAC control and TAC MR-409 mice. n = 13 for TAC control cells, n = 11 for TAC MR-409 cells. Results are mean ± SEM $*P < 0.05$.

Table S1. Primers used for RT-PCR analysis

Target gene			Primer sequence
Human			
GHRH-R (NM_000823.3)	First PCR (322 bp)	Forw	5′-TTCCTGATCCCACTCTTTGG-3′
		Rev	5′-AGCTGCCCAAATTCAAGTGT-3′
	Second PCR (191 bp)	Forw	5′-CTGCTTCCTCAACCAAGAGG-3′
		Rev	5′-AGCTGCCCAAATTCAAGTGT-3′
GHRH (NM_021081.5)	First and second PCR (175 bp)	Forw	5′-AATTGGAGAGCATCCTGGTG-3′
		Rev	5′-ACCGGTATGGGGGAATTTTA-3′
SV1 (XM_011515263)	(523 bp)	Forw	5′-TGGGGAGAGGGAAGGAGTTGT-3′
		Rev	5′-GCGAGAACCAGCCACCAGAA-3′
β-actin (NM_001101.3)	(250 bp)	Forw	5′-GGTCATCTTCTCGCGGTTGGCCTTGGGGT-3′
		Rev	5′-CCCCAGGCACCAGGGCGTGAT-3′

Table S2. Primers used for real-time PCR analysis

Target gene		Primer sequence
Human		
NPPA (NM_006172.3)	Forw	5′-CAGGATGGACAGGATTGGAG-3′
	Rev	5′-TCCTCCCTGGCTGTTATCTTC-3′
NPPB (NM_002521.2)	Forw	5′-CTTTCCTGGGAGGTCGTTCC-3′
	Rev	5′-GTTGCGCTGCTCCTGTAAC-3′
18S (KU939309.1)	Forw	5′-CGCAGCTAGGAATAATGGAATAGG-3′
	Rev	5′-CATGGCCTCAGTTCCGAAA-3′
Rat		
Nppa (NM_008725)	Forw	5′-CTGCTAGACCACCTGGAGGA-3′
	Rev	5′-AAGCTGTTGCAGCCTAGTCC-3′
Myh7 (NM_017240)	Forw	5′-ATCAAGGGAAAGCAGGAAGC-3′
	Rev	5′-CCTTGTCTACAGGTGCATCA-3′
Epac1 (XM_008765778)	Forw	5′-GAGAATGGCTGTGGGAATGT-3′
	Rev	5′-AGGGGTTCCTCATGGTTAGG-3′
18S (X_01117)	Forw	5′-CCCATTCGAACGTCTGCCCTATC-3′
	Rev	5′-TGCTGCCTTCCTTGGATGTGGTA-3′
Mouse		
Myh6 (NM_001164171.1)	Forw	5′-CGCATCAAGGAGCTCACC-3′
	Rev	5′-CCTGCAGCCGCATTAAGT-3′
Myh7 (NM_080728.2)	Forw	5′-GCATCAAGGAGCTCACC-3′
	Rev	5′-CTGCAGCCGCAGTAGGTT-3′
Nppa (NM_008725.3)	Forw	5′-GTCAGTCGTTTGGGCTGTAAC-3′
	Rev	5′-AGACCCAGGCAGAGTCAGAA-3′
Nppb (NM_001287348.1)	Forw	5′-CACAGATCTGATGGATTTCAAGA-3′
	Rev	5′-CTCATCTTCTACCGGCATC-3′
Acta1 (NM_001272041.1)	Forw	5′-CGGGAGAAGATGACTCAAA-3′
	Rev	5′-GTAGTACGGCCGGAAGCATA-3′
Caspase3 (NM_001284409.1)	Forw	5′-AGTCTGACTGGAAAGCCGAAAC-3′
	Rev	5′-CCACTGTCTGTCTCAATGCCA-3′
Bax (NM_007527.3)	Forw	5′-GGAGATGAACTGGATAGCAATATGG-3′
	Rev	5′-GTTTGCTAGCAAAGTAGAAGAGGGC-3′
Col3a1 (NM_009930.2)	Forw	5′-CCTGGCTCAAATGGCTCAC-3′
	Rev	5′-CAGGACTGCCGTTATTCCCG-3′
p53 (NM_001127233.1)	Forw	5′-GTACCTTATGAGCCACCCGA-3′
	Rev	5′-CTTCTGTACGGCGGTCTCTC-3′
18S (NR_003278.3)	Forw	5′-GTAACCCGTTGAACCCCATT-3′
	Rev	5′-CCATCCAATCGGTAGTAGCG-3′