Supplemental Materials

Materials and Methods

Animal care

The care of all animals complied with the Osaka University animal care guidelines. All animal experiments were approved by the Experimental Animal Care and Use Committee of the Graduate School of Pharmaceutical Sciences, Osaka University (approved as Douyaku 26-4) and by the Institutional Animal Care and Use Committee (IACUC) at RIKEN Kobe Branch. All experimental procedures were in accordance with the Guide for the Care and Use of Laboratory Animals, Eighth Edition, updated by the US National Research Council Committee in 2011. All mice for experiments were maintained at the Animal Care Facility of Graduate School of Pharmaceutical Sciences, Osaka University and euthanized by inhalation of isoflurane in a euthanasia chamber for experimental use.

Reagents

Reagents used in this study included isoprenaline hydrochloride, Dulbecco's modified Eagle's medium (DMEM) and foetal bovine serum (FBS) (Merck, Germany). Penicillin−streptomycin was obtained from Life Technologies (Carlsbad, CA, USA). The cDNA of the protein kinase A (PKA) catalytic subunit α (PKAcα; BC054834, IMAGE:6439034) was obtained from Dharmacon (Lafayette, CO, USA).

Surgical procedures and echocardiographic analysis

To analyse hypertrophic response to chronic β-adrenergic stimulation, Alzet osmotic mini-pumps (ALZA Corp, Mountain View, CA, USA) containing isoprenaline hydrochloride (50 mg/kg/day) were surgically inserted in the backs of β2ARKO or control mice subcutaneously. Two weeks after insertion, the mice were sacrificed and

subjected to gravimetric analysis. Two-dimensional and motion mode (M-mode) transthoracic echocardiography was performed using an iE33 model equipped with a 15-MHz transducer (Philips Electronics, Amsterdam, Netherlands) as previously described [2]. The investigator was blinded to the identity of the analysed mice.

Preparation of neonatal rat cardiomyocytes

Neonatal rat cardiomyocytes (NRCMs) were prepared and cultured as previously described [3]. In brief, ventricles excised from 1–2-day-old Wistar rats (purchased from Kiwa Laboratory Animals, Japan) were excised, minced, and digested in a solution containing 0.1% collagenase type IV (Sigma-Aldrich, St. Louis, MO, USA) and 0.1% trypsin (Thermo Fisher Scientific, Waltham, MA, USA) to obtain a single-cell suspensions. To remove non-myocytes by differential adhesion, after pre-plating the cells on culture dishes for 60–90 min at 37°C in 5% CO_2, floating cells were collected and used as NRCMs. Isolated NRCMs were cultured in DMEM(Sigma-Aldrich) containing high glucose with L-Glutamine and sodium bicarbonate (Sigma-Aldrich), supplemented with 10% FBS (Life technologies) and bromodeoxyuridine (0.1 μg/mL; Sigma-Aldrich).

Preparation of cardiac fibroblasts from adult mice

Primary cultures of adult cardiac fibroblasts were obtained from 2–3-month old mice. After injection of heparin sodium (50 units/mouse; Wako Pure Chemical, Japan), hearts were excised, minced, and digested for 45 min in buffer containing collagenase B (0.025 U/mL), collagenase D (0.025 U/mL), and proteinase type XIV (0.02 mg/mL) (Sigma-Aldrich). The digested heart samples were filtered through a 70-μm mesh followed by centrifuging for 5 min at 1000 x *g*. The cell pellet was resuspended and plated in DMEM supplemented with 10% FBS, and cardiomyocytes were removed by differential adhesion. Primary cardiac fibroblasts were used at passage 2 to avoid cardiomyocyte contamination and ensure a more homogenous population of fibroblasts.

Quantitative RT-PCR

Quantitative RT-PCR was performed according to the manufacturer's protocol. Total RNA was prepared from hearts using QIAzol reagent (QIAGEN, Germantown, MD, USA). First-strand cDNA was synthesized from 1 μg total RNA with an oligo dT primer and then used to detect the expression of each mRNA. The mRNA expression in the left ventricle (LV) was quantified by real-time RT-PCR using the Applied Biosystems StepOne Real-Time PCR system with the SYBR green system (Applied Biosystems, Carlsbad, CA, USA). As an internal control, the expression of *Gapdh* mRNA was estimated with the SYBR green system. Total RNA was also prepared from cardiac fibroblasts or myocytes using QIAzol reagent (QIAGE). First-strand cDNA was synthesized from 1 μg total RNA with an oligo dT primer, followed by amplification using specific primers of β1AR, β2AR or β3AR. The corresponding PCR products were visualized using agarose gel. Primer sequences are shown in Supplemental Table 2.

Western blotting analysis

Immunoblotting analyses were performed as described previously [2]. The primary antibodies used include anti-PKAcα, anti-PKArIα (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and anti-GAPDH (Merck Millipore) antibodies. Bound antibodies were visualized using horseradish peroxidase (HRP)-conjugated secondary antibodies (Santa Cruz Biotechnology) and ECL reagent (Promega, Madison, WI, USA), and images of probed blots were obtained by ImageQuant LAS 4010 using ImageQuant TL software (GE Healthcare, UK). The densities of corresponding bands were quantified using ImageJ software (National Institute of Health, Bethesda, MD, USA).

Histological analysis (M-T, CSA)

Hearts were excised, immediately fixed in 10% formalin (Nacalai Tesque, Japan)

containing PBS, and embedded in paraffin. Serial 5-μm heart sections were prepared and stained with Masson's trichrome, or wheat germ agglutinin (WGA)-FITC conjugate (Sigma-Aldrich). Cross-sectional areas of cardiomyocytes were analysed on WGA-FITC-stained slides using ImageJ software as previously described [4]. Fibrotic areas were quantified by measuring the blue-stained areas in seven independent images of Masson's trichrome-stained slides per heart using ImageJ software.

Immunocytochemical analysis

Neonatal rat ventricular myocytes were fixed with 4 % paraformaldehyde, and blocked with 3% bovine serum albumin. Cells were stained with anti-α actinin antibody (Sigma-Aldrich) followed by incubation with Alexa Fluor 488-conjugated goat anti-rabbit antibody (Molecular Probes, OR, USA) as a secondary antibody. Nuclear staining was performed using DAPI (Nacalai Tesque). Stained cells were observed using a fluorescence microscope (Leica TCS SP5, Leica, Wetzlar, Germany).

Scratch assays in cardiac fibroblasts

Migration of cardiac fibroblasts from the hearts of β2ARKO or control mice was assessed using an in vitro scratch assay following a previously described method [2].

Cellular hypertrophy experiments

To prepare the conditioned medium, cardiac fibroblasts obtained from the hearts of mice with the indicated genotypes were cultured until passage 2 and maintained in serum-free medium for 24 h prior to isoproterenol or vehicle administration. NRCMs were prepared and maintained under serum-free conditions for 24 h prior to the administration of conditioned media. Conditioned media were collected 48 h after isoproterenol stimulation and added to NRCMs with an equal volume of residual media. Stimulated NRCMs were cultured for 24 h after administration. To specifically visualize

cardiomyocytes, cells were stained with anti-α-actinin antibody, followed by incubation with Alexa Fluor 488-conjugated goat anti-rabbit antibody (Molecular Probes) as a secondary antibody. Those NRCMs were observed and imaged using a fluorescence microscope (FSX100, Leica), and cell surface areas were measured using ImageJ software.

References

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