Supplemental Data

Cardio-protective effects of VCP modulator KUS121 in models of myocardial infarction

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Supplemental Methods

Mice

C57BL/6J male mice were purchased from Japan SLC. Mice were maintained in temperature-controlled rooms with a 14:10 h light:dark cycle in specific pathogen-free conditions at the Institute of Laboratory Animals of Kyoto University Graduate School of Medicine. This study was approved by the Kyoto University Ethics Review Board.

Cell culture

H9C2 rat cardiomyoblast cells were cultured in Dulbecco's modified Eagle's medium (DMEM, 1% glucose) (Nacalai Tesque) supplemented with 10% fetal bovine serum (FBS) and antibiotics (Gibco™, 10378016). To induce ER stress, tunicamycin (0.1–0.2 μg/ml, Sigma-Aldrich, T7765) was added to the cultured cells. Cultured cells were counted using Countess II (Thermo Fisher Scientific).

To evaluate cellular ATP levels, an ATP assay reagent for cells (Toyo B-net, Tokyo, Japan) was added to cultured cells, and luciferase activities were measured using a plate reader (ARVO X3, PerkinElmer).

Measurements of mitochondrial respiration using an XF96 Extracellular Flux analyzer

Oxygen consumption rate (OCR) was measured using an XF96 extracellular flux analyzer (Agilent Technologies). First, H9C2 rat cardiomyoblast cells were seeded at a density of 10,000 cells/well and cultured in DMEM supplemented with 5% FBS and antibiotics for 12 hours in XF96-well plates (Agilent Technologies). Then, H9C2 cells were cultured with tunicamycin in the absence or presence of KUS121 for 6 hours. Cells were washed once with assay medium (Agilent Technologies) supplemented with 25 mM glucose (Sigma-Aldrich, G8769) and 1 mM sodium pyruvate (Gibco™, 11360070), and incubated with assay medium for 1 hour in a 37°C in a non-CO_2 incubator. Then, OCR was measured using an XF96 extracellular flux analyzer.

For measurements of mitochondrial respiration, the inhibitors were used as followed: 2 μM oligomycin (Sigma-Aldrich, 75351), ATP synthase inhibitor; 1 μM carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP) (Sigma-Aldrich, C2920), uncoupler of mitochondrial oxidative phosphorylation; a mix of 1 μM rotenone (Sigma-Aldrich, R8875) and 1 μM antimycin A (Sigma-Aldrich, A8674), complex I and III inhibitor respectively. As shown in Supplementary Fig. 2b, after the basal respiration was measured, oligomycin, FCCP, and a mix of rotenone and antimycin A, were added sequentially to measure ATP production-linked respiration, maximal respiration, and non-mitochondrial respiration, respectively. Then, proton leak (non-ATP production-linked respiration) and spare respiratory capacity were calculated using the basal respiration and these parameters.

Ischemia and reperfusion injury models in mice

The ischemia and reperfusion (I/R) injury models were as described previously (1).

Briefly, 8-week-old mice were anesthetized with sodium pentobarbital (64.8 mg/kg) administered intraperitoneally. An endotracheal tube was introduced and positive pressure ventilation was provided using a rodent respirator. After mice were fixed in a right lateral position, the thoracic cavity was opened through left thoracotomy in the third intercostal space. A 7-0 prolene suture was passed underneath the left anterior descending (LAD) coronary artery at 2 mm below the tip of the left auricle, and the LAD was ligated with a PE-10 tube. The occlusion of the LAD coronary artery was confirmed by checking for the appearance of a paler color in the anterior wall of the LV within a few seconds after ligation. After 45 min of ischemia, reperfusion was induced by untying the knot and removing the PE-10 tube. Reperfusion was confirmed by checking restoration of a red color in the anterior wall of the LV. The thoracic cavity was closed with 7-0 prolene sutures, and the skin was closed with 4-0 silk sutures.

Double staining with triphenyltetrazolium chloride and Evans blue

To evaluate the ischemic and infarcted area, double-staining using triphenyltetrazolium chloride (TTC) and Evans blue dye was performed in the I/R injury models. After 24 hours of reperfusion, mice were anesthetized, the thoracic cavity was reopened, and the LAD coronary artery was re-ligated at the same site. The heart was then perfused with 1% Evans blue dye (Wako, 054-04062), to distinguish the ischemic area, the area at risk (AAR) that was not stained with dye. The heart was excised and the left ventricle (LV) was cut into 5 transverse slices from the apex to the base. The slices were incubated in 1% TTC solution (Sigma-Aldrich, T8877) at 37°C for 15 min, photographed and weighed. For each slice, the TTC-unstained area (infarcted area), AAR, and LV area were calculated using Image J. Then, the weight of the infarcted area and AAR were calculated from each slice weight. The components of all slices were summed, and the total infarcted area weight was divided by the total AAR weight (infarcted area/AAR) for injury size, and total AAR weight divided by total LV weight (AAR/LV) for ischemic size.

Histology

After administration of an overdose of anesthetics, mice were perfused with 4% paraformaldehyde (PFA) before excising the heart, and tissue samples were further fixed in 4% PFA at 4°C overnight. The following day, the tissue samples were transferred to 70% ethanol for dehydration before embedding in paraffin. The sequential sections of the LVs were obtained at an 800 μm intervals from the point of ligation to the apex (about 4 sections per heart). Then, the sections were deparaffinized and stained with Masson's trichrome staining. Images were acquired using a microscope (BZ-9000, Keyence). We quantified the total infarcted area in LVs by measuring the infarcted area of each sections using ImageJ software.

Histological analysis was performed by an experimenter who was blinded to treatment groups.

Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick End Labeling (TUNEL)

Staining

TUNEL staining was performed to investigate the protective effects of KUS121 on cardiomyocyte apoptosis in I/R injury models. As shown in Fig. 4a, KUS121 was administered immediately after reperfusion, and the heart was excised 4 hours after reperfusion. The heart was fixed in 4% PFA at 4℃ for 24 hours, transferred to 15% sucrose solution at 4°C for 12 hours and to 30% sucrose solution at 4°C overnight, and then embedded in Tissue-Tek OCT compound (Sakura Finetech). The heart was frozen using dry ice, and cut into 7 μm-thick sections. TUNEL assay was performed using the In-Situ Cell Death Detection Kit, TMR Red (Roche Applied Science), according to the manufacturer's instructions. The sections were co-stained with anti-cardiac troponin I (1:200) (rabbit polyclonal, Abcam, ab47003) followed by Alexa Fluor 488 (1:200) (donkey anti-rabbit, Invitrogen, A21206) to identify cardiomyocytes. Nuclei were counterstained with DAPI (1 μg/mL, Dojindo, D523).

The number of apoptotic cardiomyocytes, defined as TUNEL-positive cells surrounded by troponin I in ischemia region, was counted more than 10 fields in 2 different sections per heart at x 200 magnification, and expressed as a percentage of the total cardiomyocytes.

Echocardiography

To analyze the cardiac function of mice, we performed echocardiography (Vevo®2100, VISUALSONICS) at the indicated time points after I/R injury. Mice were kept under inhalation anesthesia with 2.0% isoflurane. LV wall thickness, LV diameter, and LV function (EF and FS) were measured in M mode of the parasternal short-axis view.

Quantitative real-time PCR

To evaluate mRNA expression levels, single-strand cDNA was synthesized from 1 μg of total RNA by means of a reverse transcriptase reaction, and quantitative PCR (qPCR) was performed using a StepOnePlus™ (Thermo Fisher) with THUNDERBIRD® SYBR qPCR Mix

(TOYOBO, QPS-201). Expression levels were normalized using housekeeping genes as indicated. The primer sequences are as follows:

VCP (human) forward, 5'-CCCAGCCCAAGATGGATGAA-3';

VCP (human) reverse, 5'-CGTTTGCCGTACTTCACATCAG-3';

β-actin (human) forward, 5'-AGGCACTCTTCCAGCCTTCC-3';

β-actin (human) reverse, 5'-GCACTGTGTTGGCGTACAGG-3';

VCP (mice) forward, 5'-TTTTGACAAGGCACGGCAAG-3';

VCP (mice) reverse, 5'-GCTCCACCACCATCTCCAATA-3';

CHOP (mice) forward, 5'-AGCTGGAAGCCTGGTATGAGGA-3';

CHOP (mice) reverse, 5'-AGCTAGGGACGCAGGGTCAA-3';

GAPDH (mice) forward, 5'-AAATGGTGAAGGTCGGTGTG-3';

GAPDH (mice) reverse, 5'- AATCTCCACTTTGCCACTGC3'.

Quantification of heart ATP levels in I/R injury models of Go-ATeam2 mice

Go-ATeam2 mice express a FRET-based ATP biosensor similar to the one used for cell culture, except the fluorescent protein reporters were orange fluorescent protein (OFP) and green fluorescent protein (GFP), rather than yellow fluorescent protein (YFP) and cyan fluorescent protein (CFP), respectively (2,3) (M.Y. et al., manuscript in preparation). I/R injury models were utilized as described in *Ischemia and reperfusion injury models in mice*, above. Briefly, 8-week-old Go-ATeam2 mice were anesthetized, and an endotracheal tube was introduced for positive pressure ventilation. The thoracic cavity was opened to expose the heart, and LAD coronary artery was ligated with a PE-10 tube for 45 min. Then, reperfusion was induced by untying the knot and removing the PE-10 tube. Observations of the hearts were performed using a Leica M165 FC stereo microscope (Leica) with a 1× objective (Leica, Plan Apo 1.0) and the following DualView2 filter sets (INDEC Biosystems, Santa Clara, CA, USA): for dual emission ratio imaging, 470/40 excitation filter - dichroic mirror 540 DCLP - 515/30

for GFP and 575/40 for OFP. Images of fluorescence emission were captured every 5 minutes from pre-ischemia to 60 min after reperfusion, using ORCA-Flash4.0 (Hamamatsu, Japan). The images were obtained at 30 ms intervals for 1 second. Imaging data of OFP/GFP emission ratios were analyzed using MetaMorph (Molecular Devices, Sunnyvale, CA).

Western blotting

Western blotting was performed using standard procedures as described previously (4). *In vitro* cell lysates were collected using cell scrapers in chilled lysis buffer consisting of 100 mM Tris-HCl, pH 7.4, 75 mM NaCl and 1% Triton X-100 (Nacalai Tesque) supplemented with Complete Mini protease inhibitor cocktail (Roche, 11836153001), 0.5 mM NaF, and 10 mM Na_3VO_4 just before use. The protein concentration was determined using a BCA protein assay kit (Bio-Rad, 5000006JA). All samples (15 μg of protein) were suspended in lysis buffer, fractionated using NuPAGE 4–12% Bis-Tris Mini gels (Thermo Fisher Scientific, NP0322BOX) and transferred to Protran nitrocellulose transfer membranes (Whatman). The membrane was blocked using PBS containing 5% non-fat milk for 30 min and incubated with the primary antibody overnight at 4°C. After a washing step in PBS-0.05% Tween-20 (PBS-T), the membrane was incubated with the secondary antibody for 1 h at room temperature. The membrane was then washed with PBS-T and detected using Pierce™ Western Blotting Substrate (Thermo Fisher Scientific, NCI3106) and Pierce™ Western Blotting Substrate Plus (Thermo Fisher Scientific, NCI32132) using a LAS-4000 Mini system (Fuji Film). The primary antibodies are as follows: anti-VCP (Cell signaling technology, #2649), anti-BiP (Cell signaling technology, #3183), anti-CHOP (Santa Cruz Biotechnology, sc-575), anti-β-actin (Sigma-Aldrich, A5316). The secondary antibodies are as follows: anti-Rabbit IgG (GE Healthcare, NA934V), HRP-Linked, anti-Mouse IgG, HRP-Linked (GE Healthcare, 931V).

Ischemia and reperfusion injury models in pigs

Porcine I/R injury models were performed as described previously (5,6).

Briefly, 3-month-old pigs (35–40 kg) were anesthetized, an endotracheal tube was introduced, and positive pressure ventilation was provided with respirator. A 7Fr vascular sheath was placed in the right femoral artery for vascular access and then heparin (100 IU/kg) and amiodarone (4.3 mg/kg) were administered intravenously. During the operation, potassium (6 mEq/hr) and amiodarone (20 mg/hr) were administered continuously.

First, left ventriculography (LVG) was performed to assess cardiac function before ischemia. Then, the left main coronary artery was engaged with a 7Fr Hockey Stick guiding catheter and left coronary angiography (CAG) was performed with injection of contrast agent to identify the location of the occlusion. A 0.014-inch guidewire was inserted into the LAD coronary artery and a 3.0–20 mm over-the-wire balloon (Terumo, Japan) was advanced distal to the second diagonal branch. The balloon was inflated there and CAG was performed to confirm complete occlusion of the LAD coronary artery. After 60 mins of occlusion, reperfusion was induced by deflation of the balloon, confirmed by CAG. Then, KUS121 at a dose of 0.64, 2.5, or 5.0 mg/kg, or 5% glucose solution (as a control) was administered into the LAD coronary artery through the wire lumen of the balloon catheter. At last, LVG was performed to assess cardiac function after reperfusion, the 7Fr vascular sheath was removed, the pigs were weaned from ventilation and transferred to the animal care unit.

The dose of 0.64 mg/kg was calculated from a twelfth of the murine dose of 160 mg/kg (the same dose as administered after reperfusion in Fig. 4a) based on body surface area (7), and from coronary blood flow, accounting for 4–5% of cardiac output (8). The dose of 2.5 or 5.0 mg/kg was calculated as 4 or 8 times of the dose of 0.64 mg/kg, respectively.

Quantification of infarcted area in pigs

At 7 days after reperfusion, the pigs were anesthetized, an endotracheal tube was introduced, and positive pressure ventilation was provided with a respirator. First, gadolinium enhanced

cardiac magnetic resonance imaging (MRI) was performed to evaluate the infarcted area by late gadolinium enhancement on cardiac MRI.

Next, to evaluate the infarcted area by histology, double staining with TTC and Evans blue was performed. A 7Fr vascular sheath was placed in the right and left femoral artery for vascular access and then heparin (100 IU/kg) was administered intravenously. First, LVG was performed to assess cardiac function, and then the left and right coronary arteries (LCA and RCA) were engaged with 7Fr Hockey Stick guiding catheters and left and right CAG were performed to confirm no occlusion in the LCA and RCA. A 0.014-inch guidewire was inserted into the LAD coronary artery and a 3.0–20 mm over-the-wire balloon (Terumo, Japan) was advanced to the site of the previous occlusion. The balloon was inflated and CAG was performed to confirm complete occlusion of the LAD coronary artery. Then 60 ml of 1% Evans blue dye was injected into the LCA and 30 ml into RCA through the guiding catheters. In addition, 10 ml of 1% TTC was injected into the occluded area of the LAD coronary artery through the wire lumen of the over-the-wire balloon.

Then, the pigs were sacrificed with an intravenous injection of potassium chloride after double staining, and the heart was excised and sliced in 10-mm-thick slices from the apex to the base of the heart. The slices were submerged in 1% TTC for 15 min at 37°C. All slices were then weighed, each side of the slice was photographed and fixed with a 10% formaldehyde solution. For each slice, the TTC unstained area (infarcted area), the Evans blue unstained area (the AAR), and LV area were calculated using Image J. Then, the weight of the infarcted area and AAR were calculated from each slice weight. The components of all slices were summed and the total infarcted area weight was divided by the total AAR weight (infarcted area/AAR) for injury size and the total AAR weight divided by the total LV weight (AAR/LV) for the ischemic size.

Analysis of TTC staining and cardiac MRI was performed by an experimenter who was blinded to treatment groups.

Statistical analysis

Measured data are presented as mean ± standard error of the mean (SEM). For statistical comparisons between two groups, unpaired Student's t-test was used. For statistical analysis of three or more groups, one-way analysis of variance (ANOVA) was used. In one-way ANOVA, Sidak's post-hoc test was performed to compare all pairs of groups (Fig. 3b–f and 5b–e, and Supplementary Fig. 4b–e) and Dunnett's post-hoc test to compare one group as a control to the other groups (Fig. 2b–e and h, and 8c, d and f). A p-value of <0.05 was considered as statistically significant. Statistical analyses were performed using GraphPad Prism 6 (GraphPad Software, Inc.).

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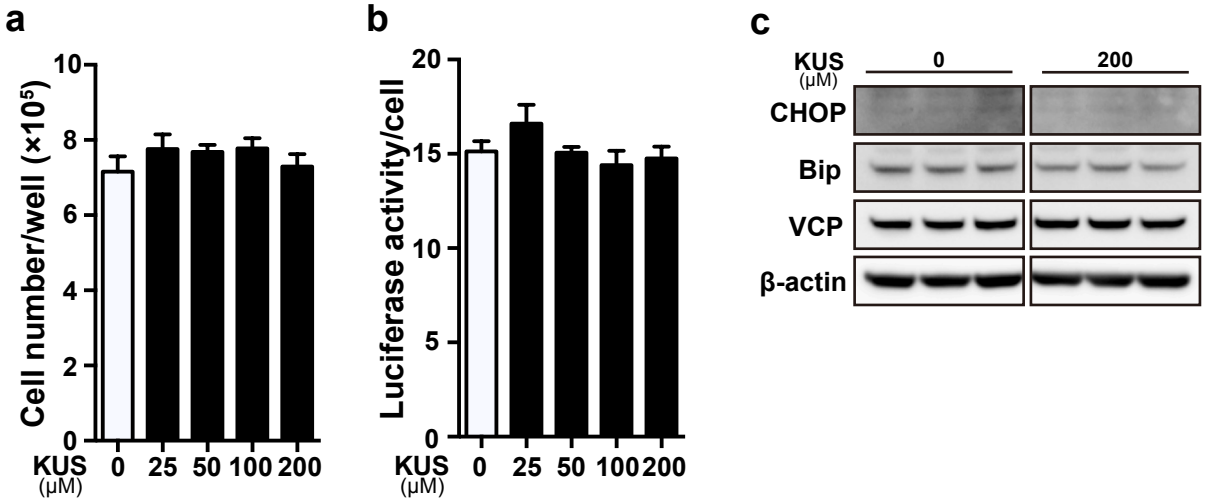
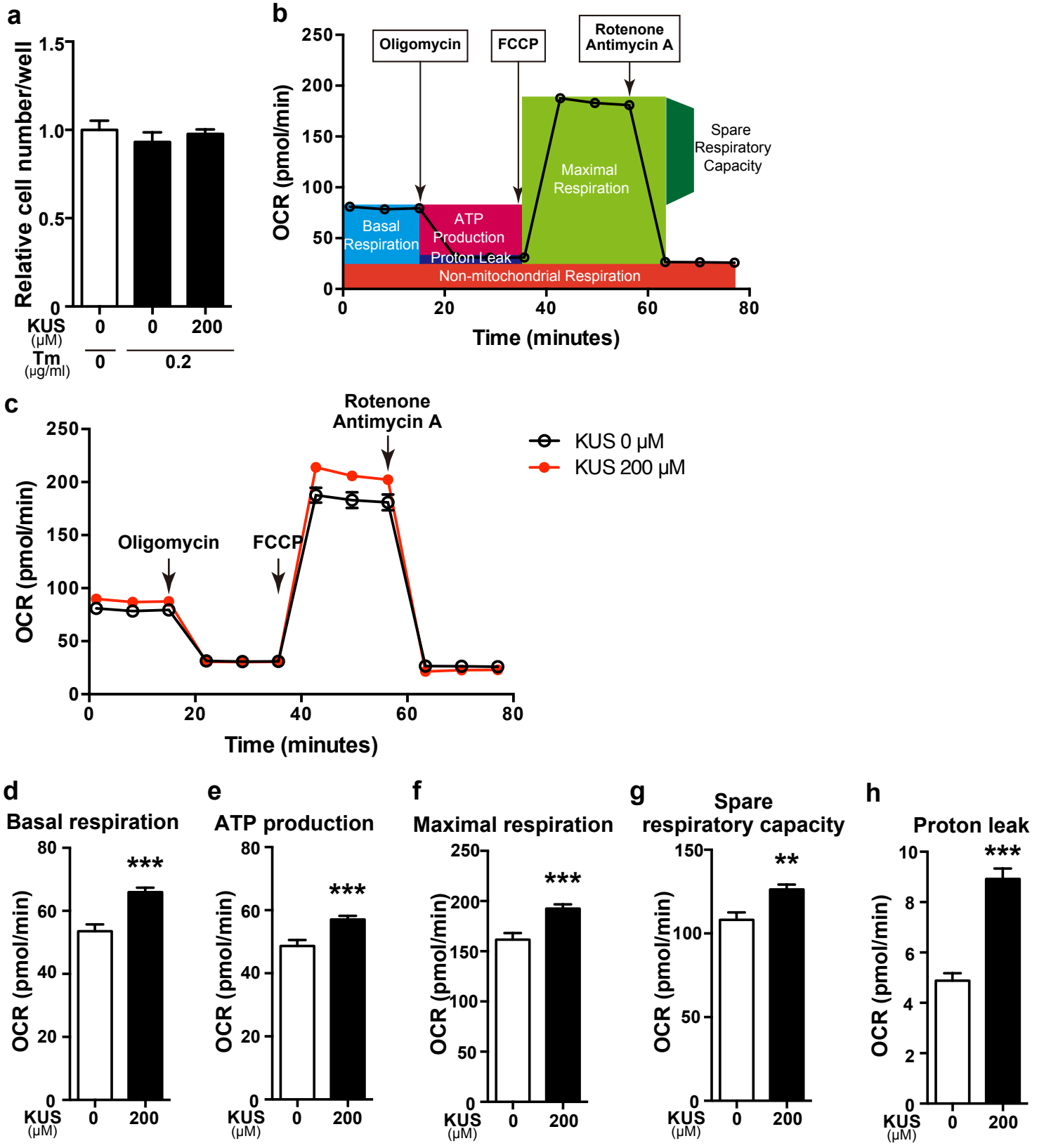


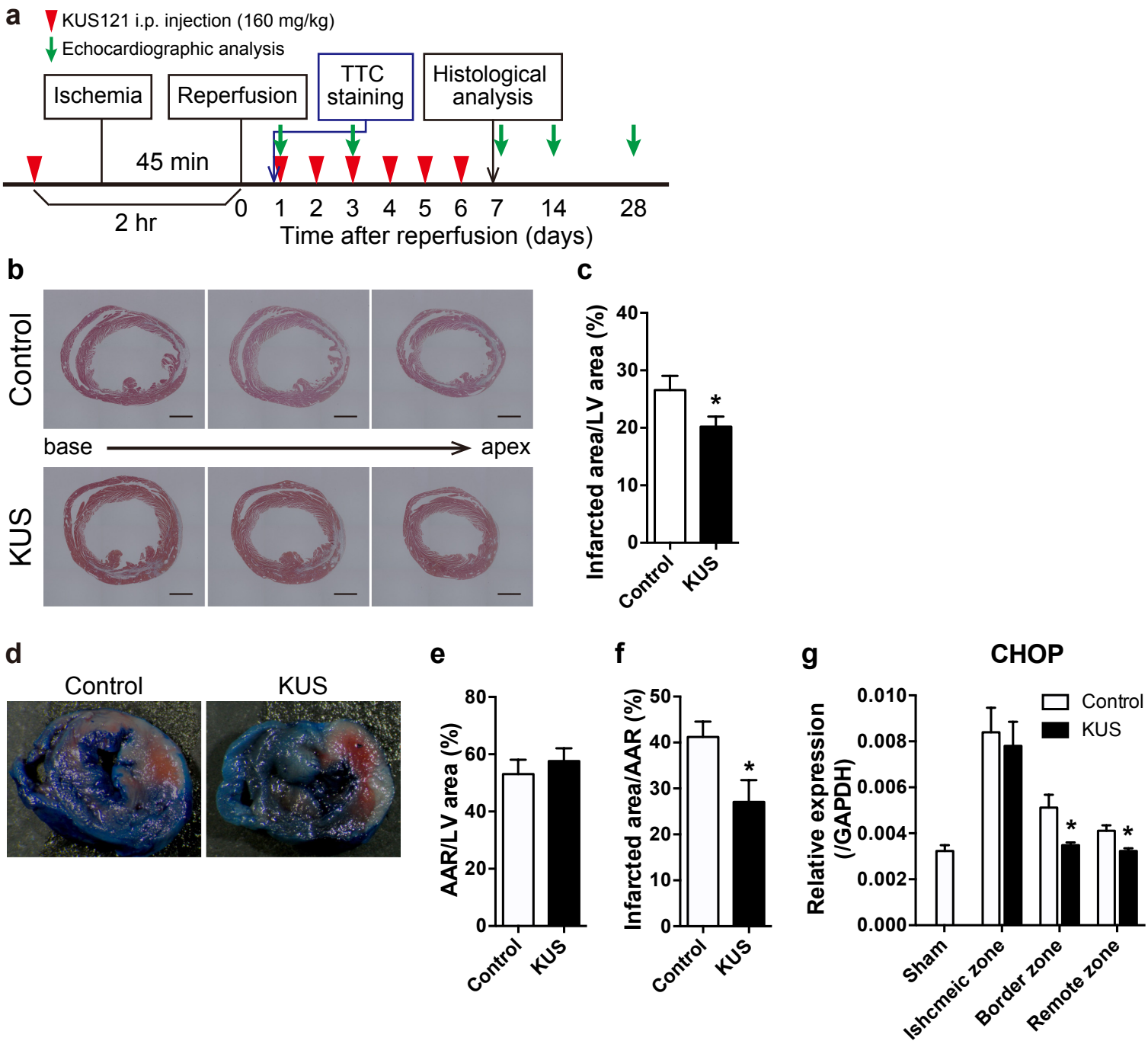
Supplementary Figure 1. KUS121 does not affect cell growth, cellular ATP levels, or protein expression levels in normal culture conditions.

(a, b) Number and ATP levels of H9C2 cells in normal culture with different concentrations of KUS121 (25, 50, 100, and 200 μM, n=3). Data are presented as mean ± SEM. (c) Western blotting analysis of H9C2 cells, cultured with and without KUS121 (200 μM, n=3).



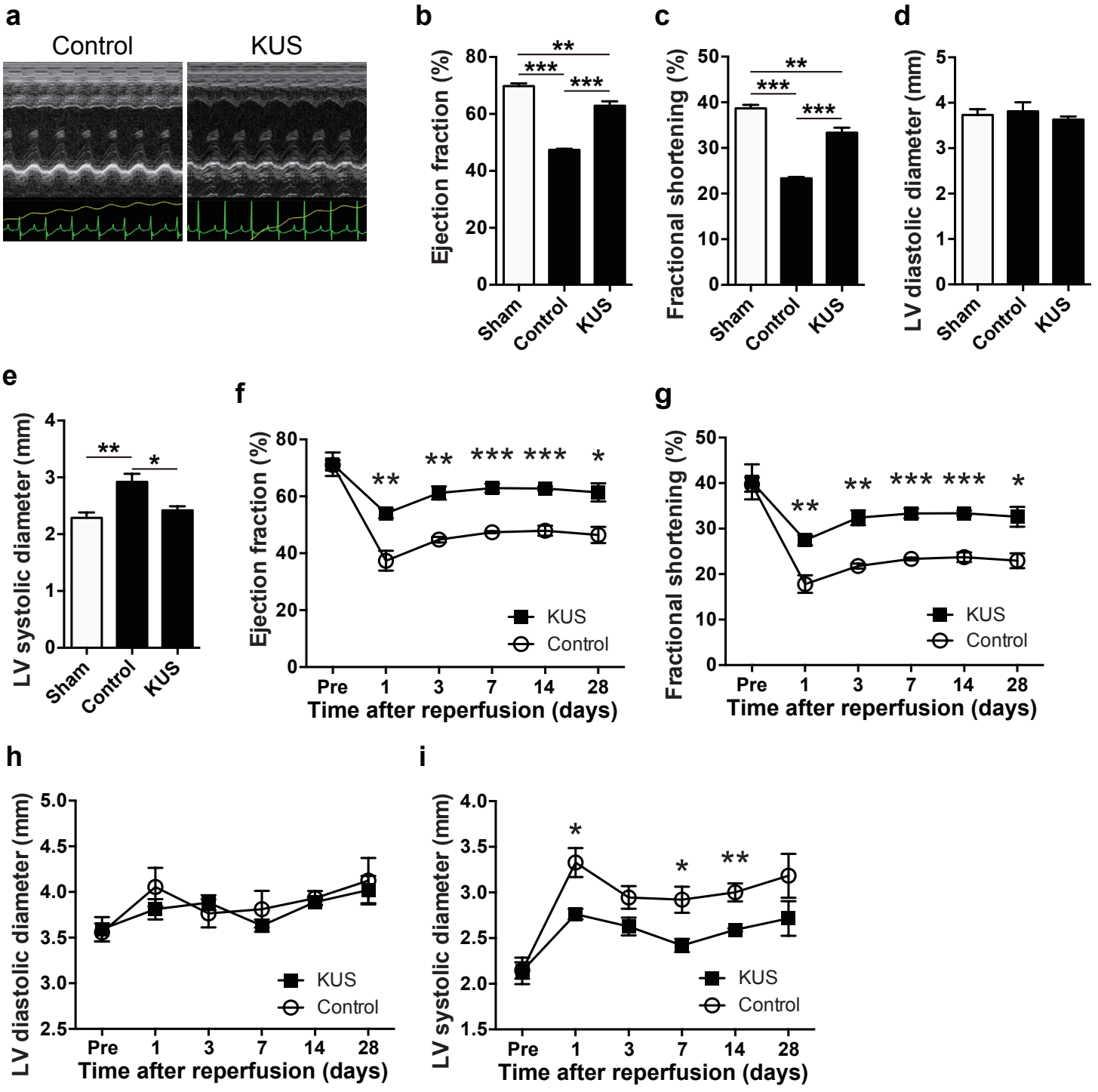
Supplementary Figure 2. KUS121 increases mitochondrial respiration in normal culture conditions.

(a) Relative number of H9C2 cells treated with tunicamycin (0.2 μg/ml) for 6 hours, with and without KUS121 (200 μM). The number of cells in normal culture conditions was the reference, indicated as 1. Data are presented as mean ± SEM. (b) Schematic diagram of oxygen consumption rate (OCR) measured using an XF96 extracellular flux analyzer. After basal OCR was measured, oligomycin (2 μM), carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP) (1 μM), rotenone (1 μM), and antimycin A (1 μM) were added sequentially to assess the parameters of mitochondrial respiration. (c) OCR in normal culture conditions. OCR at each time point was obtained from an average of 10 replicate wells and presented as mean ± SEM. (d–h) Parameters of mitochondrial respiration: basal respiration (d), ATP production-linked respiration (e), maximal respiration (f), spare respiratory capacity (g), and proton leak (h). **P<0.01, ***P<0.001, using unpaired two-tailed Student's t-test. Data are presented as mean ± SEM.



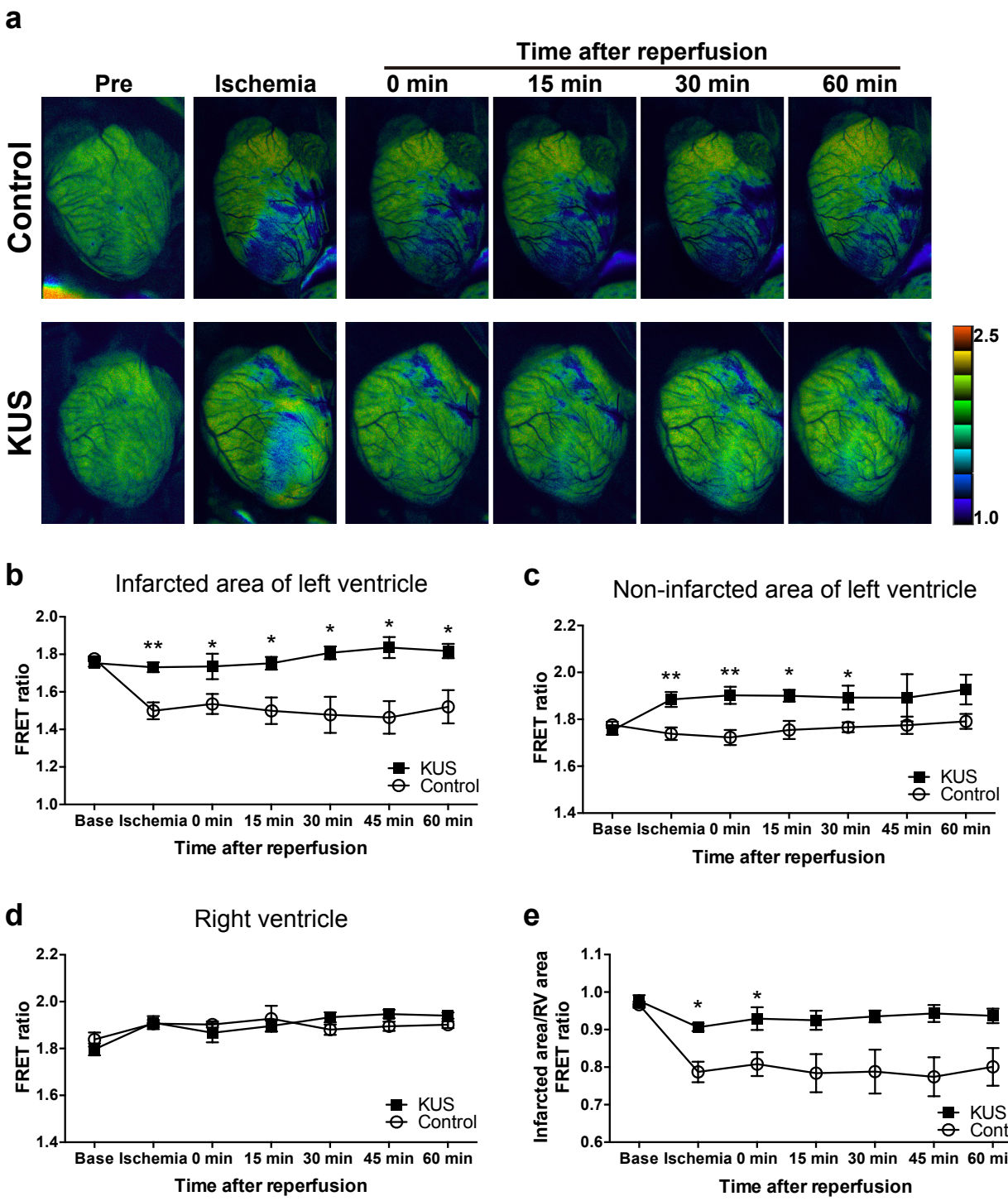
Supplementary Figure 3. KUS121 pretreatment attenuates cardiac damage in murine ischemia and reperfusion injury models.

(a) Schematic diagram of ischemia and reperfusion (I/R) injury procedures and schedules of KUS121 administration. (b) Representative images of Masson's trichrome staining in the short axis of left ventricles (LVs) at 7 days after I/R injury. Black bars indicate 1000 μm. (c) Quantification of infarcted areas in LVs at 7 days after I/R injury (Control, n=6; KUS121, n=10). *P<0.05, using unpaired two-tailed Student's t-test. (d) Representative images of double staining with triphenyltetrazolium chloride (TTC) and Evans blue in the short axis of LVs at 24 hours after I/R injury. (e, f) Quantification of infarcted area/area at risk (AAR) and AAR/LV in double staining with TTC and Evans blue (Control, n=7; KUS121, n=7). *P<0.05, using unpaired two-tailed Student's t-test. (g) Expression levels of C/EBP homologous protein (CHOP) in LVs at 1 hour after I/R injury. LVs were divided into three parts: ischemic zone, border zone, and remote zone (Sham, n=6; Control, n=12; KUS121, n=8). The expression level of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control. *P<0.05, using unpaired two-tailed Student's t-test. All data are presented as mean ± SEM.



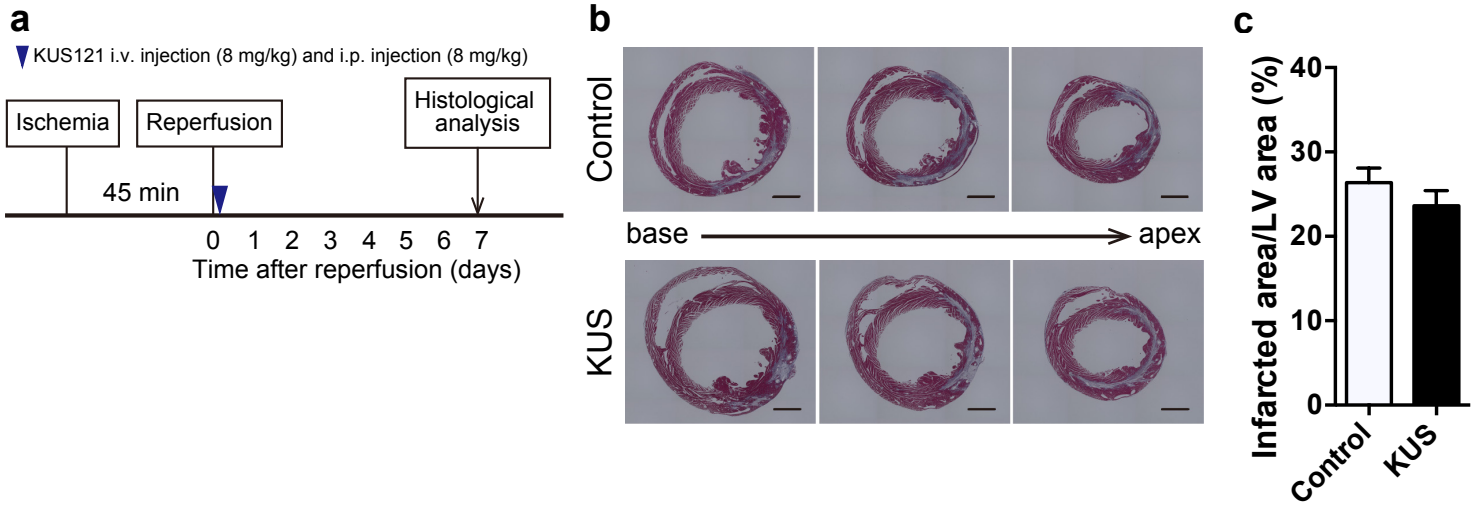
Supplementary Figure 4. KUS121 pretreatment preserves cardiac function in ischemia and reperfusion injury models.

(a) Representative images of M-mode echocardiogram of Control and KUS121-treated animals at 7 days after ischemia and reperfusion (I/R) injury. (b–e) Echocardiographic data 7 days after I/R injury. Ejection fraction (EF; b), fractional shortening (FS; c), left ventricle (LV) diastolic diameter (LVDd; d), and LV systolic diameter (LVDs; e) were measured (Sham, n=4; Control, n=3; KUS121, n=4). *P<0.05, **P<0.01, ***P<0.001, using one-way ANOVA with Sidak's post-hoc test. (f-i) Echocardiographic data at the indicated time points after I/R injury (Supplementary Fig. 3a). *P<0.05, **P<0.01, ***P<0.001, using unpaired two-tailed Student's t-test. All data are presented as mean ± SEM.



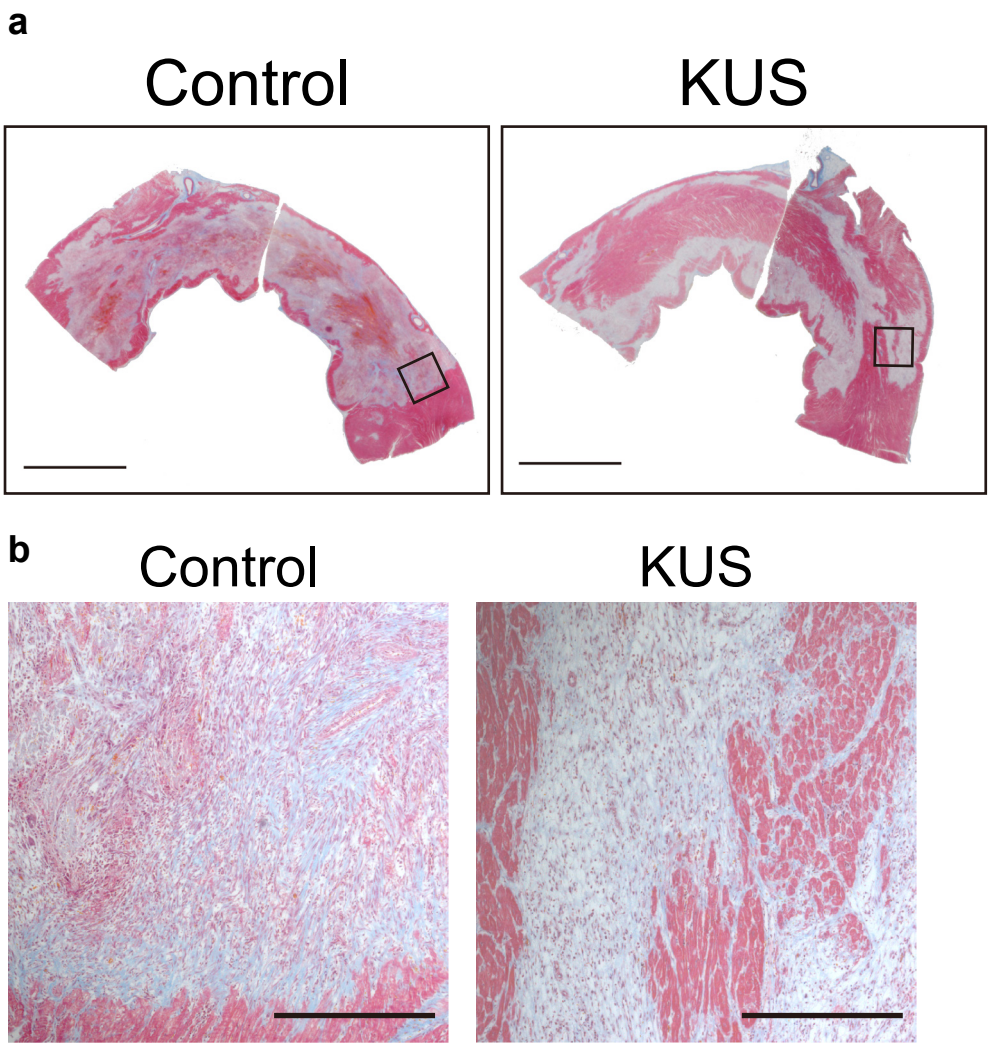
Supplementary Figure 5. KUS121 pretreatment maintains ATP levels in ischemia and reperfusion injury models.

(a) Representative pseudocolor ratiometric fluorescence resonance energy transfer (FRET) images of whole hearts in ischemia and reperfusion injury models of Go-ATeam2 mice, when KUS121 was administered before ischemia as shown in Supplementary Fig. 3a. Pseudocolor images were obtained at various time points in ischemia and after reperfusion. In pseudocolor images, warmer colors represent high FRET ratios and cooler colors low FRET ratios. (b–d) Quantification of ATP levels in infarcted areas of left ventricles (LVs) (b), in non-infarcted areas of LVs (c), and in right ventricle (RV) areas (d) by FRET ratio (Control, n=6; KUS121, n=6). *P<0.05, **P<0.01, using unpaired two-tailed Student's t-test. (e) ATP ratio of infarcted area of LV to RV area. *P<0.05, using unpaired two-tailed Student's t-test. All data are presented as mean ± SEM.



Supplementary Figure 6. Beneficial effect of KUS121 treatment is negligible at a dose of 16 mg/kg.

(a) Schematic diagram of ischemia and reperfusion (I/R) injury procedures and schedules of KUS121 administration. (b) Representative images of Masson's trichrome staining in the short axis of left ventricles (LVs) at 7 days after I/R injury. Black bars indicate 1000 μm. (c) Quantification of infarcted area in LVs at 7 days after I/R injury (Control, n=15; KUS, n=16). Data are presented as mean ± SEM.



Supplementary Figure 7. The histology of Masson's trichrome staining is similar to that of triphenyltetrazolium chloride staining in porcine ischemia and reperfusion injury models.

(a) Representative whole images of the infarcted area in the same slices as shown in Fig. 9b. Black bars indicate 1000 mm. (b) Enlarged images of black squares shown in (a). Black bars indicate 500 μm.