Supplemental Materials

Cardiac μ-receptor contributes to opioid-induced cardioprotection in chronic heart failure

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Expanded Materials and Methods

Post-myocardial infarction heart failure model

Post-myocardial infarction heart failure was induced by ligation of the left anterior descending coronary artery (LAD), as previously described with minor modifications.[1] Adult male Sprague-Dawley (SD) rats were subjected to left thoracotomy under anaesthesia with pentobarbital sodium (60 mg kg BW^{-1}, IP., Sigma, USA), and ligation of the LAD was performed by a 4-0 silk thread. The animals were monitored daily after surgery and cardiac function was assessed using echocardiography under light isoflurane anaesthesia 8 weeks after coronary artery ligation. A left thoracotomy without LAD ligation was undertaken in the sham group (normal) (n=6 in each group).

Echocardiography

At the end of 8^{th} week, rats from normal (NS-injected or sham) and failing (DOX-treated or post-infarction) groups were subjected to echocardiography to measure left ventricular ejection fraction (LVEF) and left ventricular fractional shortening (LVFS). Echocardiography was also performed before DOX injection (0 week), and 1, 8 weeks after DOX injection (n=6 at each time point). Rats were anesthetized with 1.5% isoflurane and subjected to echocardiography by an individual blinded researcher who specialized in cardiac ultrasound. Two-dimensional and M-mode echocardiography was performed using ACUSON Sequoia 512 with 15L8W-S transducer (Siemens,

Germany). Left ventricular end-diastolic diameter (LVEDD) and left ventricular end systolic diameter (LVESD) were measured and LVEF and LVFS were automatically calculated by the echocardiography system.

Histological examination

At the end of 8th week, heart sections of 4 rats from NS and DOX-treated groups were subjected to histological examination. Heart tissues were fixed in 10% neutralized formalin. The fixed samples were embedded in paraffin and sectioned into serial sections (5μm) and then routinely stained with hematoxylin-eosin according to conventional procedures. The sections were examined and photographed under a light microscope (×200).

Plasma BNP level

Blood samples were collected from NS and DOX-treated rats before sacrificed at the end of 8^{th} week for detection of plasma brain natriuretic peptide (BNP) levels by ELISA kit (Abcam, USA) (n=10 in each group).

Absolute quantitative RT-PCR

To detect the copy number of μ-opioid receptor mRNA, the total RNA extracted from normal and failing rat hearts (n=6 in each group) was subjected to absolute quantitative RT-PCR. At first, RNA from rat midbrain was reverse transcribed and amplified by PCR in the presence of the primers of μ-receptor or β-actin, as well as dNTP, Taq buffer, $MgCl_2$ and Taq enzyme. The PCR amplified products of μ-receptor and β-actin were separated on a 2% agarose gel and purified, and then cloned into a pMD® 18-T Vector (TaKaRa, China). After that, the vector was extracted with a SanPrep column plasmid DNA extraction kit (Sangon Biotechnology, China) followed by DNA sequencing (DNA-seq). The concentration and quantity of plasmid DNA was determined by

spectrophotometry, and the copy numbers were calculated based on the total DNA and their molecular weights. Ten-fold serial dilutions of known amounts of μ-receptor and β-actin plasmid DNA were used as PCR standards. The reverse transcribed cDNA of normal and failing rat hearts were amplified using the SYBR PrimeScript^{TM} RT-PCR kit as described before. The quantity of μ-receptor and β-actin mRNA in the samples was calculated based on the data obtained from the standard curves.

Relative quantitative RT-PCR

Total RNA was extracted from rat ventricular myocardium of normal (NS) or failing (DOX) rats at the end of 8th week (n=12 in each group) for relative quantitative RT-PCR analysis. In the DOX-treated group, heart tissues were also separately collected before DOX injection (0 week), and 1, 8 weeks after DOX injection (n=6 at each time point) for mRNA detection.

A total of 400 ng RNA was reverse transcribed to cDNA and amplified using the SYBR PrimeScript^{TM} RT-PCR kit (TaKaRa Biotechnology, China). The primers of rat β-actin gene (for normalization) and μ-opioid receptor gene were designed and synthesized by Sangon Biotechnology (Shanghai, China) as follows: β-actin, forward: 5'-CCCATCTATGAGGGTTACGC-3', reverse: 5'-TTTAATGTCACGCACGATTTC-3'; μ-opioid receptor, forward: 5'-ATCGGGCTCCAAAGAAAAGG-3', reverse: 5'-CATCCAGGAAGGCGTAAAGAAC-3'. The PCR analysis was performed with an ABI Prism 9700HT system (Foster City, CA, USA) according to the manufacturer's instructions. The PCR cycling conditions were set as follows: 95°C for 30 s followed by 40 cycles of denaturation at 95 ℃ for 5 s and annealing/extension for 30 s at 60 °C. Threshold cycles (Ct) were automatically calculated by the PCR system and relative gene expression levels were calculated by the $2^{-\Delta\Delta CT}$. In each experimental run, double-distilled water served as the

negative control. All amplification reactions were performed in triplicate for each sample, and dissociation curves were generated after each run to confirm the amplification of specific transcripts.

Western blot analysis

Total proteins were extracted from rat ventricular myocardium of normal and failing rat hearts for detecting μ-receptor protein expression (*n*=6 in each group). In Langendorff perfusion experiments, tissue samples (*n*=4 in each group) were taken from left ventricular myocardium 10 min after reperfusion and total proteins were extracted for detecting kinases proteins.

The proteins were quantified by bicinchoninic acid (BCA) protein assay (Pierce, USA). Equal amount of proteins (50 μg) was subjected to 10% SDS-PAGE gel followed by electrotransfer onto polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA, USA). Nonspecific antibody binding was blocked in Tris-buffered saline solution/0.1 Tween 20 (TBST) containing 5% skimmed milk. Membranes were incubated overnight at 4℃ with primary antibodies against μ-opioid receptor (Abcam, Cambridge, UK), p-ERK1/2($Thr^{202}/^{204}$), ERK1/2 (Cell Signaling Technology, USA) or p-GSK-3β (Ser^{9}), GSK-3β (Abcam, Cambridge, UK) and β-actin (Abcam, Cambridge, UK), respectively. After that, the membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies for 1 hour at room temperature. After washing three times with TBST, the membranes were covered with ECL (Amersham Pharmacia Biotech, USA) and detected by KODA X-ray film or autoradiography (Tanon Science & Technology, China). Densitometric analysis of protein bands was performed by an independent blinded observer with Image J2x software (NIH). The relative expressional levels of phosphorylated protein were normalized to their total protein as well as the loading control β-actin protein.

Radioligand binding assay

The μ-receptor specific binding sites in membrane preparations of normal and failing rat hearts (*n*=10 in each group) were identified using radioligand binding assay. Tissues from left ventricular myocardium were immediately chilled in ice-cold assay buffer (50 mM Tris-HCl, 1 mM EGTA, 5 mM $MgCl_2$, pH 7.4), homogenized, and centrifuged at 48,000×g at 4 ℃ for 20 min to obtain membrane rich fractions, then resuspended in assay buffer followed by 10 min incubation at 37 ℃ to remove endogenous ligands. The membrane homogenates were centrifuged again and resuspended in assay buffer, aliquoted and stored at -80 ℃. The radioligand binding experiments were performed using the radio-labeled specific μ-receptor agonist [^{3}H] [D-Ala2, N-MePhe4, Gly5-ol] enkephalin (DAMGO) (49.2Ci·mmol^{-1}, PerkinElmer, USA). Membrane protein (100 μg) was incubated with10 nM (close to the saturation concentration) [^{3}H] DAMGO for 30 min at 37 ℃ in binding buffer [50 mM Tris–HCl, 5 mM EDTA, 5 mM $MgCl_2$, 100 mM NaCl, 0.2 % bovine serum albumin (BSA)]. Nonspecific binding was defined as radioactivity remaining bound in the presence of 10 μM unlabeled DAMGO (Tocris, USA) and subtracted from the total binding. Reactions were stopped by rapid filtration through a Brandel MLR-24 harvester (Brandel, Gaithersburg, MD), and bound radioligand was collected on GF/C filtermats (Whatman, Kent, UK) and rinsed three times with ice-cold 50mM Tris-Hcl, pH 7.4. Filters were dried, saturated with EcoLume scintillation cocktail (MP Biomedicals, Solon, OH) and radioactivity was counted using a Wallec 1450 MicroBeta counter (PerkinElmer, Waltham, MA).

Immunohistochemical analysis

A portion of normal and failing rat heart tissues from ventricular myocardium (n=4 in each group) were fixed in buffered formalin and embedded in paraffin followed by cutting into 4 μm sections for immunohistochemical analysis. Paraffin sections were deparaffinized in xylene and rehydrated.

Antigen retrieval was done by heating the sections in 0.01M sodium citrate buffer (pH 6.0) to 160 °C for 2 min. After cooling, the sections were rinsed in Tris-buffered saline (TBS) and incubated in methanol 3% H_2O_2 for 15 min to deactive endogenous peroxidase activity. After rinsed with TBS, the sections were incubated in 10% goat serum to block nonspecific binding and then incubated with the primary antibody of rabbit anti-μ-opioid receptor (dilution: 1:50) overnight at 4 °C in a humid chamber. Following that, the sections were rinsed in TBS and incubated with preoxidase-labeled polymer conjugated to goat anti-rabbit immunoglobulin for 20 min at room temperature. The immunostaining was developed by using a 3,3'-diaminobenzidine (DAB) kit (Zhongshan Goldenbridge Biotechnology, China).

Double immunofluorescence staining

Cardiac tissues from left ventricle of normal and heart failure rats (n=4 in each group) were embedded in tissue-Tek compound (OCT, Miles Inc. Elkhart, IN), frozen, and cut into 10 μm thick sections using a cryostat. The sections were then incubated overnight with the rabbit polyclonal anti-μ-opioid receptor (Abcam, Cambridge, UK) in combination with mouse anti-α-SA antibody (Abcam, Cambridge, UK) or whole serum guinea pig anti- TRPV1 antibody (Neuromics, Edina, MN, USA). After incubation with primary antibodies, the tissue sections were washed with PBS and then incubated with Texas Red conjugated goat anti-rabbit antibody (Vector Laboratories) in combination with Alexa Fluor 488 goat anti-mouse antibody (Invitrogen, Karlsruhe, Germany) or Alexa Fluor 488 goat anti-guinea pig antibody (Invitrogen, Karlsruhe, Germany). Thereafter, sections were washed with PBS, and the nuclei stained bright blue with 4’-6-diamidino-2-phenylindole (DAPI, 0.1 $\mu g \cdot ml^{-1}$ in PBS) (Sigma, USA). Finally, the tissues were washed in PBS, mounted in Vectashield (Vector Laboratories), and imaged on a confocal laser scanning microscope, Leica SP5-DMI6000 (Leica,

Germany).

Preparation of Langendorff isolated heart perfusion

All rats were anesthetized with pentobarbital sodium (60 mg kg BW^{-1}, IP., Sigma, USA) and the hearts were quickly excised and immediately suspended in the Langendorff apparatus and perfused retrograde with K-H solution which was oxygenated with 95% O_2 and 5% CO_2 mixture and kept pH at 7.4. Meanwhile, the solution temperature was stabilized at 37 ℃ by temperature-regulating device. A 5-0 silk tread was passed LAD between the pulmonary artery and the left atrial appendage. Regional ischemia was achieved by ligation LAD and was confirmed by region cyanosis and decreased coronary flow. A latex balloon connected to a pressure transducer was inserted into the left ventricle cavity to continuously monitor hemodynamics including heart rate (HR) and left ventricular developed pressure (LVDP) by Power Lab System (AD Instrument, Australia) during the experiments. Hemodynamics parameters were continuously monitored and recorded at baseline, 30min of ischemia, and 120 min of reperfusion.

Langendorff experimental protocols

To investigate whether the activation of cardiac μ-receptor could reduce myocardial I/R injury, the normal or DOX-induced failing rat hearts were randomly divided into control and DAMGO (μ-receptor specific agonist) groups (n=10 in each group). To further examine whether the μ-receptor antagonist could block morphine (Mor) or remifentanil (Remi) preconditioning-induced cardioprotection, all rats with DOX-induced heart failure were randomly divided into 5 groups: Control, Morphine (Mor), CTOP (μ-receptor antagonist)+Mor, Remifentanil (Remi), CTOP+Remi and CTOP alone (n=10 in each group). To determine the role of ERK pathway in μ-receptor-induced cardioprotection, all rats with DOX-induced heart failure were randomly

divided into Control, DAMGO, DAMGO+PD98059 (PD, ERK specific inhibitor), and PD98059 (PD) alone groups (*n*=10 in each group).

We also compared different inhibitory effects of cardiac μ-, δ- and κ-receptor antagonists on remifentanil-induced cardioprotection in normal and DOX-induced failing rat hearts. Normal and failing rat hearts were randomly divided into Control, Remi, CTOP (μ-receptor antagonist)+Remi, naltrindole (NTD, δ-receptor antagonist)+Remi, nor-binaltorphimine (BNI, κ-receptor antagonist)+Remi groups, and NTD, BNI or CTOP alone groups (*n*=6 in each group).

Determination of infarct size

At the end of reperfusion, LAD was re-occluded and 0.25% Evans blue was injected to differentiate the area at risk (AAR) and normal area which was stained blue. The heart was frozen at -80 ℃ for 20 min and cut into 2 mm slice along the longitudinal heart axis. The slices were placed in separate vials and incubated for 10 min with 1.0 % triphenyltetrazolium chloride (TTC) (Sigma, USA) stain in phosphate buffer (pH 7.4) at 37 ℃. Then tissues were stored in vials of 10 % formaldehyde overnight to enhance the contrast of the stain and infarct size (IS) was *white*, but AAR was *brick red*. The volumes of left ventricle and right ventricle (LV + RV), AAR, IS were calculated by a blinded investigator using Image J2x software (NIH), The IS was expressed as a percentage of the AAR (IS/AAR).

Lactate dehydrogenase assay

The coronary effluent was collected to detect the activities of lactate dehydrogenase (LDH) at baseline, 5 min and 10 min after reperfusion, in order to evaluate the extent of myocardial injury. The LDH activity was assayed spectrophotometrically using an LDH kit (Nanjing Jiancheng Bioengineering, Nanjing, China). Values are expressed as international units (IU) per liter.

Supplemental Table 1

Effects of DAMGO on hemodynamics parameters in normal and DOX-induced failing hearts

	Baseline	Ischemia	Reperfusion
HR（beats min^{-1}）			
Failing			
Control	255±15	244±21	222±20
DAMGO	245±9	219±18	199±10
Normal			
Control	280±8	236±17	220±10
DAMGO	283±8	245±14	232±11
LVDP（KPa）			
Failing			
Control	13.70±0.40*	5.19±0.53	6.38±0.27
DAMGO	14.76±0.80$^{\#}$	6.25±0.80	6.12±1.20$^{\#}$
Normal			
Control	18.35±0.67	6.25±1.06	8.25±0.53
DAMGO	18.62±0.67	7.32±1.20	9.31±0.67

Data are expressed as mean± S.E.M. (n=6). Baseline: 15 min after stabilization; Ischemia: 30 min after regional ischemia; Reperfusion: 120 min after reperfusion; HR: heart rate; LVDP: left ventricular developed pressure. * $P<0.01$ as compared with the Normal Control group at the same time point; $^{\#}$ $P<0.05$ as compared with the Normal DAMGO group at the same time point by Two-way repeated ANOVA followed by Bonferroni's test.

Supplemental Table 2

Effects of CTOP on hemodynamics parameters in DOX-induced failing hearts

	Baseline	Ischemia	Reperfusion
HR（beats·min^{-1}）			
Control	255±15	244±21	222±20
Mor	245±10	228±5	190±7
CTOP+Mor	236±9	175±5[a]	167±6[*]
Remi	239±9	222±22	200±10
CTOP+Remi	290±27	252±24	207±22
CTOP	236±13	190±18[*]	171±7
LVDP（mmHg）			
Control	13.70±0.40	5.19±0.53	6.38±0.27
Mor	13.97±0.40	5.85±0.40	7.58±0.93
CTOP+Mor	13.30±0.53	5.72±1.46	5.72±0.93
Remi	15.03±0.93	5.57±0.93	5.85±1.33
CTOP+Remi	13.70±0.40	5.19±0.93	5.45±0.53
CTOP	14.23±0.40	8.91±1.20[*]	8.11±1.33

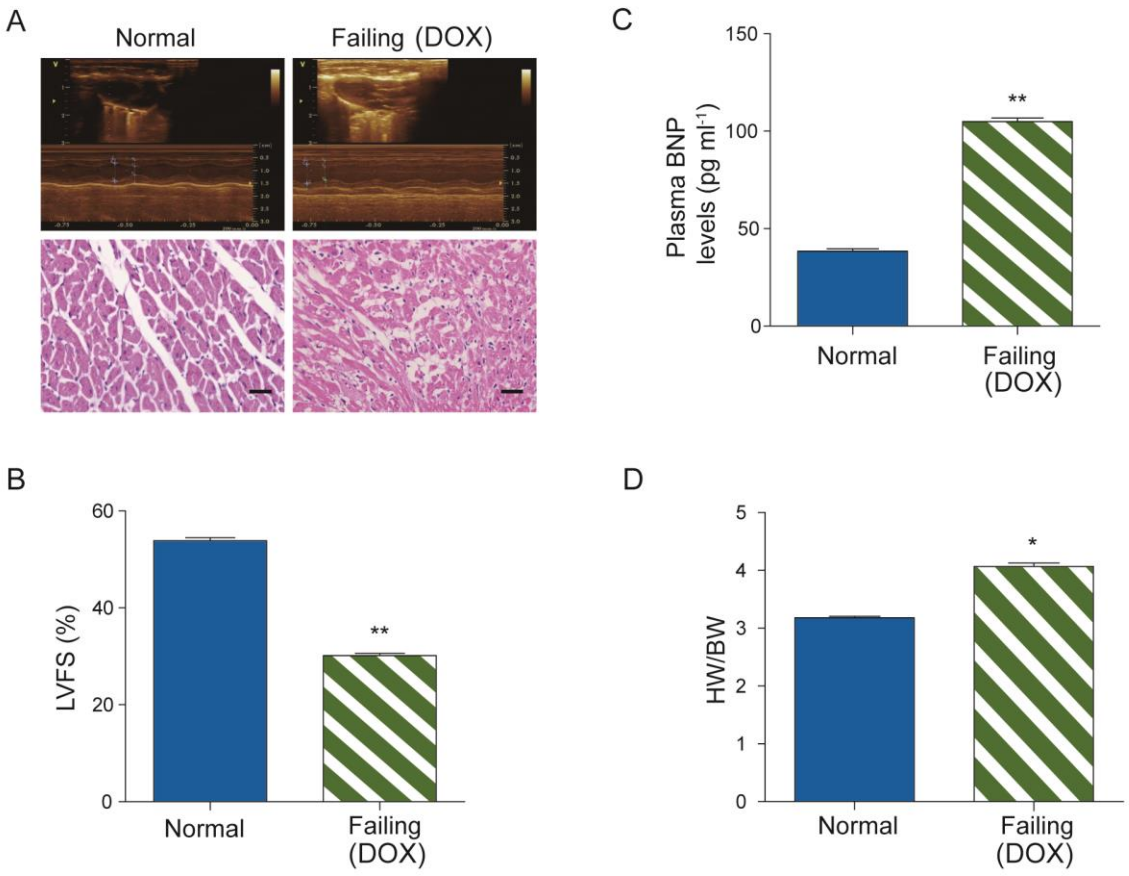
Data are expressed as mean± S.E.M. (n=6). Baseline: 15 min after stabilization; Ischemia: 30 min after regional ischemia; Reperfusion: 120 min after reperfusion; HR: heart rate; LVDP: left ventricular developed pressure. [*]P<0.05 as compared with the Control group at the same time point by Two-way ANOVA followed by Bonferroni's test.

Supplemental Table 3

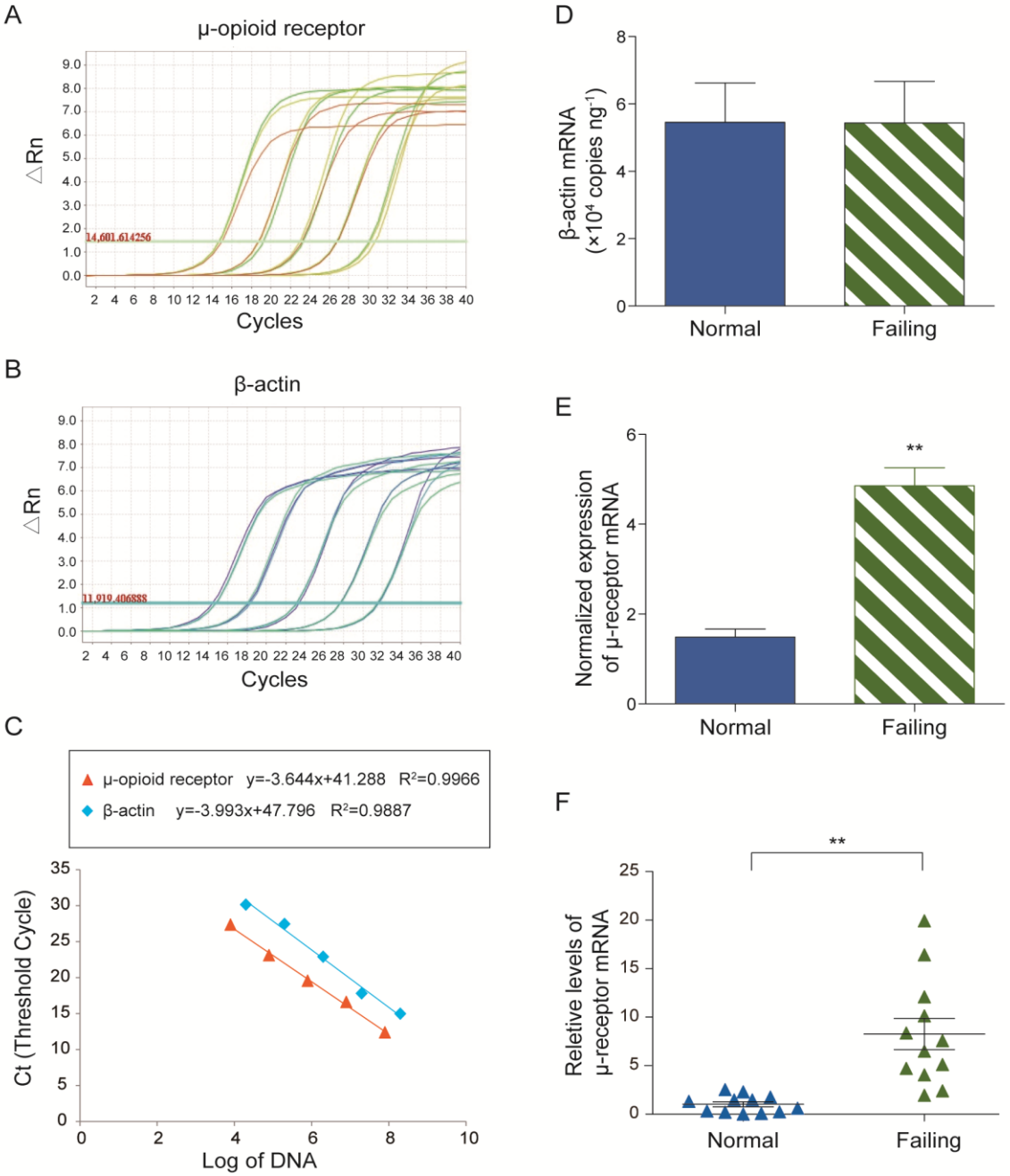
Effects of PD98059 on hemodynamics parameters in DOX-induced failing hearts

	Baseline	Ischemia	Reperfusion
HR（beats min^{-1}）			
Control	260±18	248±20	221±25
DAMGO	256±15	221±16	201±17
DAMGO+PD	268±17	219±18	186±35
PD	251±28	223±22	181±25
LVDP（mmHg）			
Control	13.43±0.80	5.59±0.93	6.52±0.53
DAMGO	15.16±0.67	6.65±0.80	6.52±1.20
DAMGO+PD	13.70±1.06	7.05±1.20	6.65±0.93
PD	14.90±1.20	6.78±1.46	6.62±0.87

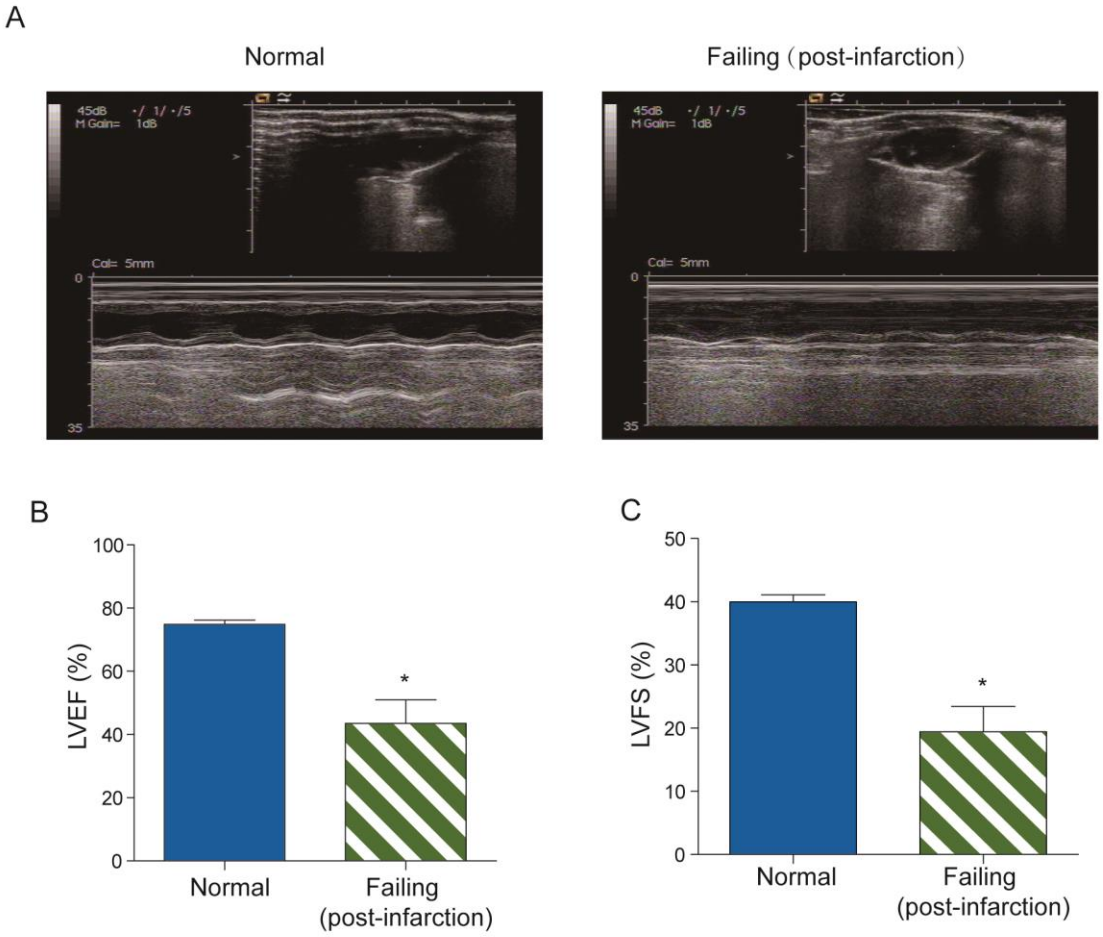
Data are expressed as mean± S.E.M. (n=6). Baseline: 15 min after stabilization; Ischemia: 30 min after regional ischemia; Reperfusion: 120 min after reperfusion; HR: heart rate; LVDP: left ventricular developed pressure.



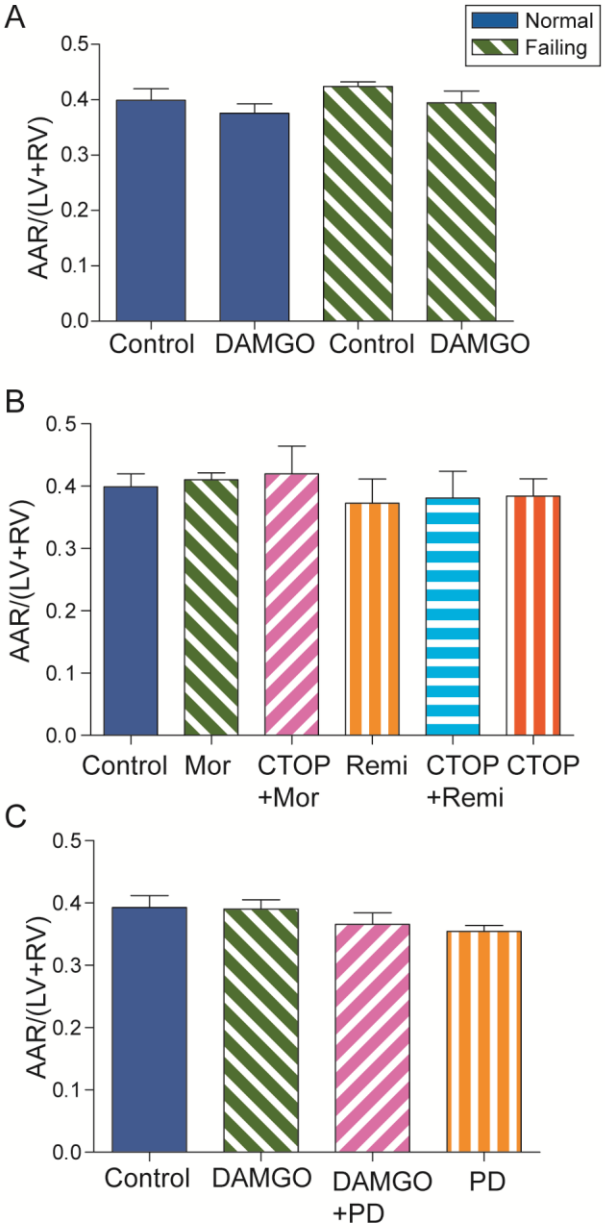
Supplemental Fig. 1 Confirmation of chronic heart failure induced by doxorubicin in rats. (**A**) Representative echocardiographic images (the *upper* lane) and hematoxylin-eosin stained heart sections (the *bottom* lane) from normal saline (NS, Normal) and doxorubicin (DOX, Failing) injected rats at the end of 8th week. Bar=100 μm. (**B**) left ventricular fractional shortening (LVFS) were automatically calculated by the echocardiography system. (n=20). (**C**) Plasma brain natriuretic peptide (BNP) levels were detected by ELISA method (n=10). (**D**) The ratio of heart weight (HW) to body weight (BW) was calculated at the end of 8th week. (n=20). ** P<0.01, *P<0.05 as compared with the Normal group by Unpaired Student's *t* test.



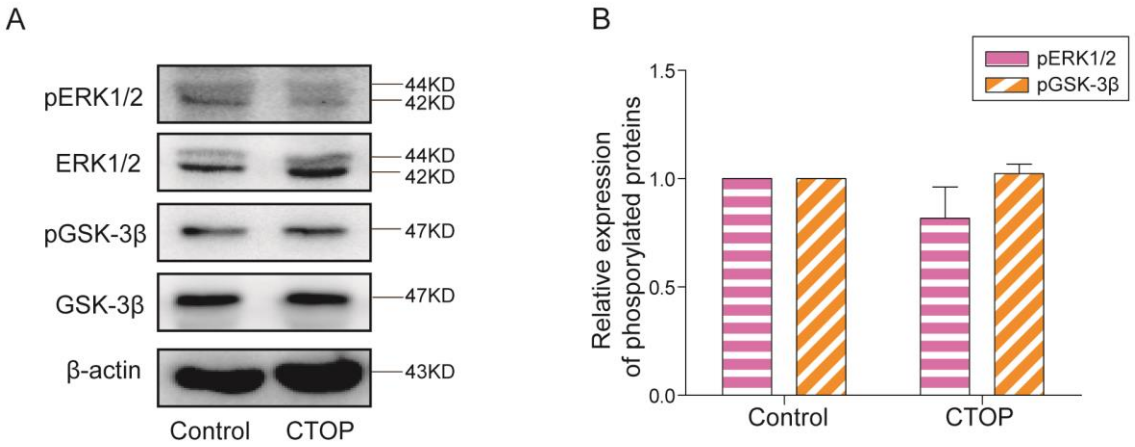
Supplemental Fig. 2 Detection of the mRNA levels of cardiac μ-opioid receptor by **quantitative RT-PCR.** Ten-fold serial dilutions of the known amounts of (**A**) μ-opioid receptor and (**B**) β-actin plasmid DNA were amplified by quantitative RT-PCR. (**C**) Linearity and correlation coefficients (R^2) of the μ-opioid receptor and β-actin standard curves. (**D**) The copy numbers of β-actin mRNA in normal and failing hearts. (**E**) The relative level of μ-receptor mRNA normalized to β-actin mRNA (n=6). (**F**) The fold changes of cardiac μ-receptor mRNA levels were detected by relative quantitative RT-PCR (n=12). **P<0.01 as compared with the Normal group by Unpaired Student' *t* test.



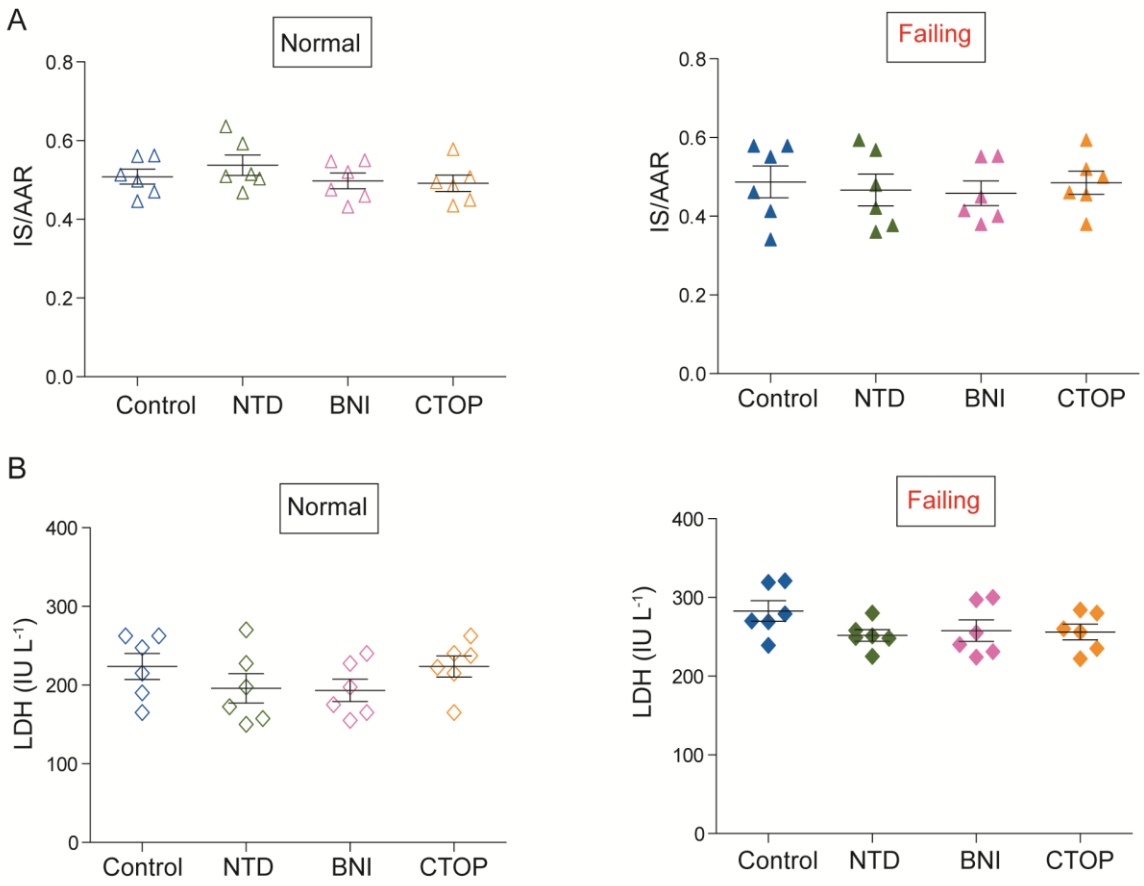
Supplemental Fig.3 Cardiac function in rats with post-myocardial infarction heart failure. (**A**) Representative echocardiographic images from sham (Normal, *left column*) and post-infarction (Failing, *right column*) rats at the end of 8th week. (**B**) Left ventricular ejection fraction (LVEF) and (**C**) left ventricular fractional shortening (LVFS) were automatically calculated by the echocardiography system (n=6). *P<0.05 as compared with the Normal group by Unpaired Student's *t* test.



Supplemental Fig. 4 Comparison of the area at risk among groups. (**A**) The ratios of AAR / (LV + RV) between control and DAMGO groups in normal and failing hearts. (**B**) The ratios of AAR / (LV + RV) among morphine, remifentanil and CTOP treated groups in failing hearts. (C) The ratios of AAR / (LV + RV) among DAMGO and PD98059 treated groups in failing hearts. AAR, area at risk; LV, left ventricle; RV, right ventricle; DAMGO, the μ-opioid receptor agonist; Mor, morphine; Remi, remifentanil; CTOP, the μ-opioid receptor antagonist.



Supplemental Fig. 5 Effects of CTOP on the phosphorylation levels of ERK and GSK-3β in failing hearts. (**A**) The μ-receptor antagonist CTOP alone did not significantly affected the phosphorylation levels of ERK1/2 and GSK-3β. (**B**) The relative expression of p-ERK1/2 or p-GSK-3β was normalized to total ERK1/2 or total GSK-3β and β-actin. The value in control group was assigned as 1. Each bar graph represents the average of mean ±S.E.M. from 4 rats.



Supplemental Fig.6 Effects of three opioid antagonists on myocardial I/R injury

(**A**) Infarct size (IS/AAR) and (**B**) LDH activities caused by I/R injury were not affected by the μ-receptor antagonist CTOP, δ-receptor antagonist NTD or κ-receptor antagonist BNI when given alone in either normal (*left lane*) or failing (*right lane*) hearts (n=6).