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Original Article

Cardioprotective effects of chloroquine pretreatment on ischemic and reperfusion injury via activation of ERK1/2 in isolated rat hearts

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Abstract

Purpose

Several therapeutic agents have been found to prevent myocardial ischemic and reperfusion (I/R) injury after cardiac surgery; however, no drug is routinely used to afford cardioprotective benefits in clinical settings. Herein, we aimed to determine whether chloroquine (CQ) pretreatment attenuates I/R injury after global ischemia in isolated rat hearts and elucidate mechanisms underlying the effects of CQ.

Methods

Isolated rat hearts were subjected to 30-min global ischemia, followed by 60-min reperfusion with Krebs-Henseleit buffer (KHB). Immediately before ischemia, 10 mL of pretreatment solutions (KHB, $n = 4$ or KHB + CQ [100 μ M], $n = 4$) were injected through the aortic root. Cardiac function was examined based on the rate pressure product (RPP). Myocardial apoptosis was evaluated using TUNEL staining. To assess the reperfusion ischemia salvage kinase pathway, protein expression levels of AKT and extracellular signal-regulated kinase (ERK1/2) were determined using western blotting. To investigate the role of ERK1/2, an ERK1/2 selective inhibitor was used in eight additional rats.

Results

The recovery rate of the RPP was higher in the KHB + CQ group than in the KHB group 60 min after I/R (KHB, $44 \pm 3\%$ vs. KHB + CQ, $69 \pm 7\%$; $P = .019$, $d = 2.2$). CQ pretreatment reduced apoptosis and enhanced the phosphorylation of ERK1/2; however, AKT phosphorylation was unaltered. In addition, the ERK1/2 inhibitor abolished CQ-mediated cardioprotective effects.

Conclusions

CQ pretreatment showed protective effects on cardiac function after I/R by activating ERK1/2.

Keywords

Chloroquine, Extracellular signal-regulated kinase, Ischemic and reperfusion injury, Cardioprotection

Introduction

Ischemic and reperfusion (I/R) injury remains a critical issue during cardiovascular surgery, potentially inducing cardiac dysfunction post-cardiac surgery and graft failure after heart transplantation. The use of cardioplegia and heart preservation solutions is a basic strategy for preventing I/R injury. In addition to modifying the solution, pretreatment with cardioprotective substances has been previously examined [1, 2]. However, no pharmacological pretreatment strategy is routinely employed for cardioprotection based on clinical evidence.

Chloroquine (CQ), an inhibitor of autophagy, is clinically used as an antimalarial and anti-inflammatory drug. Based on experimental reports, CQ can attenuate I/R injury in several organs. For instance, acute injection of CQ can reduce serum creatinine and tubular necrotic score in renal I/R injury [3]. Moreover, CQ preconditioning protects neurons from cytotoxicity and apoptosis induced by oxygen-glucose deprivation [4]. However, there are no reports on the effect of CQ on I/R injury after global myocardial ischemia. The purpose of this study was 1) to investigate whether CQ pretreatment attenuates I/R injury after global ischemia in isolated rat hearts and 2) to elucidate the mechanisms underlying the CQ-mediated effects on cardiac function after I/R.

Methods

Animals

In the present study, 10-week-old male Sprague-Dawley rats (body weight, 300–330 g) were obtained from Sankyo Labo Service Corporation and maintained under the following conditions: temperature, 22–24 °C; humidity, 40–60%; light-dark cycle, 12/12 h.

Isolated Heart Preparation

Rats were euthanized by administering intraperitoneal sodium pentobarbital (150 mg/kg; Nacalai Tesque).

After laparotomy, heparin (1000 IU/kg; Mochida Pharmaceutical Co., Ltd.) was injected into the inferior vena cava to prevent blood coagulation. The hearts were rapidly removed and trimmed in ice-cold Krebs-Henseleit buffer (KHB). Isolated hearts were perfused using the Langendorff system (LaboSupport). The prepared KHB (pH 7.4) was composed of the following substances (mM; Sigma-Aldrich): NaCl 128, KCl 5.0, MgSO₄ 1.3, KH₂PO₄ 1.0, CaCl₂ 2.5, NaHCO₃ 15, and glucose 5.0. The buffer was oxygenated with 95% O₂ and 5% CO₂. The perfusion pressure was maintained at 76 mmHg, while the chamber temperature was controlled at 37 °C with a warmed outer chamber.

Experimental Protocol

Figure 1 shows the experimental protocol. All rat hearts were first stabilized using KHB for 30 min. Subsequently, the hearts were subjected to 30 min of global ischemia and 60-min reperfusion with KHB. Immediately before ischemia, 10 mL of pretreatment solutions (KHB, n = 4 or KHB + CQ, n = 4) were injected through the aortic root at a constant pressure of 60 mmHg at 37 °C. Based on titration results shown in **Supplemental Fig. 1**, the CQ solution (chloroquine diphosphate; Wako) was used at a concentration of 100 µM. After assessment of cardiac function during reperfusion, the hearts were removed from the Langendorff system. The left ventricle of the apex side was fixed in 3.5% neutral formalin, embedded in paraffin, and sectioned at 5-µm thickness; the other side was immediately frozen in liquid nitrogen and stored at -80 °C.

Measurement of Cardiac Function

A balloon was inserted into the left ventricle through the left atrium to obtain pressure data, which were recorded with PowerLab (ADInstruments) and analyzed using LabChart (ADInstruments). The end-diastolic pressure of the left ventricle was adjusted to 5–10 mmHg. The cardiac functional parameters were as follows: coronary flow (CF), heart rate (HR), left ventricular developed pressure (LVDP), maximal rates

of increase and decrease in velocity of left ventricular pressure (+dp/dt and -dp/dt), and rate pressure product ($RPP = HR \times LVDP$). Baseline values of cardiac functional parameters were recorded at the end of the 30-min stabilization period. Inclusion criteria for baseline CF and LVDP were a flow rate ≥ 8 mL/min and developed pressure ≥ 100 mmHg, respectively. The recovery rate for each cardiac function parameter was calculated during reperfusion as a percentage of the baseline value.

TUNEL Staining

To detect myocardial cell apoptosis, a terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) assay was performed. Briefly, formalin-fixed sections were deparaffinized and rehydrated in an ethanol concentration gradient. The sections were permeabilized for 15 min in proteinase K buffer and incubated with 3% H_2O_2 for 5 min, TdT buffer (TaKaRa) for 70 min, and anti-FITC horseradish peroxidase (HRP)-conjugated antibody solution (TaKaRa) for 30 min. The chromogenic reaction was visualized using diaminobenzidine (Agilent Technologies). Then, nuclei were counterstained with hematoxylin. Ten frames were randomly selected and analyzed using ImageJ software (National Institutes of Health, Bethesda, MD). The TUNEL index (%) was calculated as the number of TUNEL-positive cells divided by the total number of cells.

Enzyme-Linked Immunosorbent Assay

To determine the effect of CQ on the I/R-induced inflammatory response, the level of tumor necrosis factor- α (TNF- α) was analyzed using an enzyme-linked immunosorbent assay (ELISA). TNF- α in frozen myocardial tissue was assayed using an ELISA kit (BioVision Inc.), according to the manufacturer's instructions. TNF- α levels were expressed as picograms of TNF- α per milligram of protein.

Western Blotting

The reperfusion ischemia salvage kinase (RISK) pathway is an important molecular factor that prevents I/R injury. To assess the RISK pathway in two parallel cascades, we examined protein expression levels of AKT and extracellular signal-regulated kinase (ERK) [5]. Autophagy has also been associated with I/R injury [6]. Autophagy is an intracellular process during which the autophagosome fuses with a lysosome to degrade unnecessary proteins, and CQ has been shown to inhibit fusion by increasing the lysosomal pH [7]. To assess autophagy, we evaluated the expression of two autophagy markers, microtubule-associated protein light chain 3 (LC3) and p62/Sequestosome 1 (p62). LC3-II and p62 are markers of autophagosome formation and autophagic degradation, respectively [8].

The protein concentration in myocardial tissue lysates was determined using the Bradford protein assay. Briefly, protein samples (25 µg) were separated using 10% sodium dodecyl polyacrylamide gel electrophoresis (Mini-PROTEANTGXTM, Bio-Rad) and blotted onto PVDF membranes (Millipore). The running and blotting of the protein were performed using the Mini-PROTEAN Tetra System (Bio-Rad). Then, membranes were blocked with an ECL blocking agent (Cytiva) and incubated with rabbit primary antibodies against AKT (1:1500; Cell Signaling [CS]), pAkt (Ser473) (1:1500; CS), ERK1/2 (1:1200; CS), pERK1/2 (Thr202/Tyr204) (1:2000; CS), LC3 (1:500; Abcam), p62 (1:10000; CS), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1:1000; CS) at 4 °C overnight. HRP-conjugated secondary antibodies (1:2000, anti-rabbit IgG; CS) were used at room temperature for 1 h. The bands were detected using a chemiluminescence system (ECL Plus, Cytiva) and semi-quantified using JustTLC (SWEDAY). GAPDH was used as a loading control.

ERK Inhibition

To evaluate whether CQ mediates cardioprotection through ERK1/2 activation, 100 nM SCH 772984 (an ERK1/2 selective inhibitor; Cayman Chemical) was mixed with the pretreatment solution for eight additionally isolated rat hearts.

Statistical Analysis

Data values are presented as mean \pm standard error of the mean (SEM). The calculated sample size was determined as $n = 4$ to detect a 20% mean difference in the recovery rate of RPP (power, 80%; $\alpha < .05$; software, G*Power). Student's t-test was used for the comparison between two groups. Tukey's post-hoc test was performed if there was a significant interaction with two-way ANOVA [9]. Statistical significance was defined as $P < .05$. The effect size (Cohen's d) was calculated to evaluate differences. The effect size was reported as very large if $d \geq 1.3$ [10]. Statistical analyses were performed using GraphPad Prism version 9.0 (GraphPad Software, Inc.).

Results

Cardiac Function

No significant differences in baseline cardiac functional parameters were detected between the KHB and KHB + CQ groups (**Supplemental Table 1**).

Figure 2 presents a comparison of recovery rates for cardiac functional parameters after I/R between the KHB and KHB + CQ groups. Sixty minutes after I/R, CF (KHB, $43 \pm 6\%$ vs. KHB + CQ, $61 \pm 10\%$; $P = .16$) and HR (KHB, $67 \pm 6\%$ vs. KHB + CQ, $75 \pm 6\%$; $P = .37$) did not differ between the two groups. The recovery rates of LVDP (KHB, $68 \pm 8\%$ vs. KHB + CQ, $91 \pm 3\%$; $P = .034$, $d = 1.9$), $+dp/dt$ (KHB, $52 \pm 3\%$ vs. KHB + CQ, $91 \pm 7\%$; $P = .002$, $d = 3.7$), $-dp/dt$ (KHB, $47 \pm 4\%$ vs. KHB + CQ, $74 \pm 8\%$; $P = .019$, $d = 2.3$), and RPP (KHB, $44 \pm 3\%$ vs. KHB + CQ, $69 \pm 7\%$; $P = .019$, $d = 2.2$) were higher in the KHB + CQ group than in the KHB group 60 min after I/R.

Apoptosis in Myocardial Tissue

Figure 3 compares the TUNEL index in myocardial tissues between the KHB and KHB + CQ groups. The

TUNEL index was lower in the KHB + CQ group than in the KHB group after I/R (KHB, $8.3 \pm 1.2\%$ vs. KHB + CQ, $4.8 \pm 0.6\%$; $P = .044$, $d = 1.8$).

Activation of Proteins Related to the RISK pathway

Figure 4 presents a comparison of the protein phosphorylation rates of AKT (**Fig. 4a**) and ERK1/2 (**Fig. 4b**) in myocardial tissues between the KHB and KHB + CQ groups. The phosphorylation rate of AKT did not differ between the two groups (KHB, 0.24 ± 0.01 vs. KHB + CQ, 0.24 ± 0.01 ; $P = .72$). The phosphorylation rate of ERK1/2 was higher in the KHB + CQ group than in the KHB group after I/R (KHB, 0.78 ± 0.05 vs. KHB + CQ, 1.03 ± 0.07 ; $P = .029$, $d = 2.0$).

Autophagy and Inflammatory Markers

Figures 5a and b present comparisons of LC3-II (**Fig. 5a**) and p62 (**Fig. 5b**) protein expression in myocardial tissues between the KHB and KHB + CQ groups. The protein expression of LC3-II (KHB, 1.1 ± 0.1 vs. KHB + CQ, 1.1 ± 0.1 arbitrary unit; $P = .77$) and p62 (KHB, 0.18 ± 0.02 vs. KHB + CQ, 0.20 ± 0.03 arbitrary unit; $P = .54$) did not differ between the two groups. **Figure 5c** presents a comparison of TNF- α expression levels in myocardial tissue between the KHB and KHB + CQ groups. The level of TNF- α did not differ between the two groups (KHB, 70 ± 7 vs. KHB + CQ, 61 ± 6 pg/mg protein; $P = .32$).

Effects of SCH 772984 on ERK Phosphorylation and CQ-Mediated Cardioprotection

To evaluate whether CQ affords cardioprotection by activating ERK1/2, a selective ERK1/2 inhibitor (SCH 772984) was added to the pretreatment solution of the additional eight isolated rat hearts. No significant differences in the baseline cardiac functional parameters were detected between the KHB, KHB + CQ, KHB + SCH 772984, and KHB + CQ + SCH 772984 groups (**Supplemental Table 2**). **Figure 6a–c** shows the effects of SCH 772984 on the protein expression of total ERK1/2 (**Fig. 6a**), pERK1/2 (**Fig. 6b**), and the

phosphorylation rate of ERK1/2 (**Fig. 6c**). The protein expression of total ERK1/2 was unaltered following SCH 772984 application in the KHB or KHB + CQ groups. In contrast, the protein expression of pERK1/2 decreased following SCH 772984 application in both groups. SCH 772984 treatment reduced the ERK1/2 phosphorylation rate in the KHB + CQ group; however, this rate was unaltered in the KHB group.

Figure 6d–f shows the effects of SCH 772984 on the recovery rate of +dp/dt (**Fig. 6d**), -dp/dt (**Fig. 6e**), and RPP (**Fig. 6f**) at 60 min post-reperfusion. The recovery rates of all parameters were reduced following SCH 772984 treatment in the KHB + CQ group; however, the values were not significantly altered in the KHB group.

Figure 6g shows the effects of SCH 772984 on the apoptosis. TUNEL index was $8.3 \pm 1.2\%$, $4.8 \pm 0.6\%$, $7.7 \pm 0.9\%$, $8.8 \pm 1.0\%$ in KHB, KHB + CQ, KHB + SCH, KHB + CQ + SCH, respectively (CQ, $P = .234$; SCH, $P = .099$; interaction, $P = .033$). Although the TUNEL index was not altered in the KHB group, it significantly increased following SCH 772984 treatment in the KHB + CQ group ($P = .0495$).

Discussions

In the present study, we demonstrated that CQ pretreatment attenuated I/R injury after global ischemia and activated ERK1/2 in the isolated rat heart. The effects of CQ pretreatment on cardiac function are dependent on ERK1/2 activation.

The effect of CQ pretreatment on cardiac function is dependent on ERK1/2 activation, a cascade of the RISK pathway. The RISK pathway is a combination of two parallel cascades, the MEK1/2-ERK1/2 and PI3K-AKT pathways, which play important roles in cell survival against I/R-induced injury [5]. ERK1/2 and AKT are members of the mitogen-activated protein kinase family, which regulates cell proliferation and differentiation in response to interaction with tyrosine kinase and G-protein-coupled receptors [11].

Several studies have reported ERK1/2-mediated cardioprotective effects in myocardial ischemia. In an intermittent hypoxia (IH) preconditioned rat myocyte model, IH preconditioning was shown to reduce the infarct size by activating ERK1/2, while AKT was not involved [12]. In *in vivo* rat hearts, preconditioning with CQ (oral administration from three days before surgery) could reduce infarct size via ERK1/2 phosphorylation [13]. However, some differences were observed in our study. First, we administered CQ via the aortic root immediately before global ischemia. Our results following CQ pretreatment warrant further experimental studies on cardioplegia and heart preservation solutions. Second, we demonstrated that CQ pretreatment improved cardiac function after I/R injury. Third, we used an ERK1/2 selective inhibitor (SCH 772984), while previous researchers inhibited MEK1/2 upstream of ERK1/2. Thus, our study provides direct evidence indicating the involvement of ERK1/2 in CQ pretreatment-mediated effects.

However, the mechanism of ERK1/2 expression in cardiac protection against I/R injury remains unclear. Reportedly, transforming growth factor- β 1 can attenuate cardiomyocyte apoptosis after ischemia and reoxygenation by activating ERK1/2 [14]. In the present study, CQ pretreatment reduced the number of apoptotic cells after I/R, and the ERK inhibitor abolished the CQ-mediated effect on apoptosis. Further studies are needed to investigate the mechanism of the CQ-mediated reduction in apoptosis.

In the present study, CQ pretreatment did not affect the expression of inflammatory markers. CQ exerts anti-inflammatory effects by downregulating TNF- α and decreasing cytokine production in mice [15]. We speculate that the underlying reasons for this discrepancy could be differences in the method of CQ administration (topical vs. systemic administration) and experimental models employed (*ex vivo* vs. *in vivo*).

Herein, CQ pretreatment did not alter autophagy markers (LC3-II and p62) at the end of 60-min reperfusion. Typically, CQ impairs the fusion of autophagosomes and lysosomes, followed by an increase in the number

of autophagosomes (LC3-II). In growth factor-stimulated liver cells, LC3-II serves as a scaffold for ERK1/2 [16]. In the present study, given that LC3-II levels did not differ between the KHB and KHB + CQ groups, we postulate that autophagy would not be associated with the activation of ERK1/2. However, the levels of autophagy markers may increase at the other time points (e.g., just after pretreatment, during and at the end of ischemia). Further study is necessary to assess the timeline of the levels of autophagy markers.

This study had several limitations. First, we did not examine the influence of CQ on ion channels in cardiomyocytes. CQ blocks inward rectifying potassium currents, rapid delayed rectifying potassium currents, sodium currents, and L-type calcium current [17]. This may also affect cardiac function after I/R (e.g., by attenuating calcium overload). Further studies are needed to investigate the effects of CQ on intracellular ion dynamics. Second, the cardiotoxicity of CQ (i.e., prolonged QT interval time and arrhythmia) should be noted [18]. Although arrhythmia was not evident in the CQ group (data not applicable), further investigations using *in vivo* models are necessary for human application. Lastly, because we did not wash out the pretreatment solution, we cannot know whether CQ during pretreatment or ischemia affected the cardioprotective effects against I/R.

Conclusions

CQ pretreatment attenuated I/R injury after global ischemia in isolated rat hearts. The effects of CQ pretreatment on cardiac function are dependent on ERK1/2 activation. Further studies are necessary to determine the clinical application of CQ pretreatment.

Statements and Declarations

Funding

The authors have no funds, grants, or other support.

Competing of Interests

The authors declare that they have no conflicts of interest.

Data Availability

The data sets generated during the current study will be shared on reasonable requests to the corresponding author.

Author Contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Ryota Murase and Yasushige Shingu. Manuscript revision was performed by Satoru Wakasa. The first draft of the manuscript was written by Ryota Murase and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Ethical Approval

All procedures were approved by the National University Corporation Hokkaido University Animal Research Committee and consistent with the Guide for the Care and Use of Laboratory Animals, published by the US National Institute of Health (NIH publication No. 85-23, revised 1996).

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Figure Legends

Fig. 1 Experimental protocol. CQ, chloroquine; KHB, Krebs-Henseleit buffer

Fig. 2 Recovery rate in cardiac functional parameters after I/R in the KHB and KHB + CQ groups: (a) coronary flow; (b) heart rate; (c) LVDP; (d) +dp/dt; (e) -dp/dt; (f) RPP. Values are mean \pm SEM. N = 4 for each group. * P < .05, ** P < .01. CQ, chloroquine; KHB, Krebs-Henseleit buffer; LVDP, left ventricular developed pressure; RPP, rate pressure product

Fig.3 TUNEL index in myocardial tissue in the KHB and KHB + CQ groups: (a) representative pictures of TUNEL staining. The arrows indicate TUNEL positive cells. (b) TUNEL index. Values are mean \pm SEM. N = 4 for each group. * P < .05. CQ, chloroquine; KHB, Krebs-Henseleit buffer

Fig. 4 Protein phosphorylation rate of AKT (a) and ERK1/2 (b) in myocardial tissue in the KHB and KHB + CQ groups. Values are mean \pm SEM. N = 4 for each group. * P < .05. CQ, chloroquine; ERK, extracellular signal-regulated kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; KHB, Krebs-Henseleit buffer

Fig. 5 Protein expression of autophagy and inflammatory markers in myocardial tissue in the KHB and KHB + CQ groups: (a) LC3-II; (b) p62; (c) TNF- α . Values are mean \pm SEM. N = 4 for each group. CQ, chloroquine; ERK, extracellular signal-regulated kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; KHB, Krebs-Henseleit buffer; LC3, microtubule-associated protein light chain 3; TNF- α , tumor necrosis factor- α

Fig. 6 Effects of SCH 772984 on ERK phosphorylation and recovery rate of cardiac function: (a)

representative bands and quantitative analysis of total ERK1/2; (b) quantitative analysis of pERK1/2; (c) phosphorylation rate of ERK1/2; (d) +dp/dt; (e) -dp/dt; (f) RPP; (g) TUNEL index. Values are mean \pm SEM. N = 4 for each group. * P < .05, ** P < .01. CQ, chloroquine; ERK, extracellular signal-regulated kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; KHB, Krebs-Henseleit buffer; RPP, rate pressure product; SCH, SCH 772984

Supplemental fig. 1 Recovery rate of RPP with different concentrations of CQ. Values are mean \pm SEM. N = 4 for each group. * P < .05, ** P < .01. CQ, chloroquine; KHB, Krebs-Henseleit buffer; RPP, rate pressure product

Fig. 1

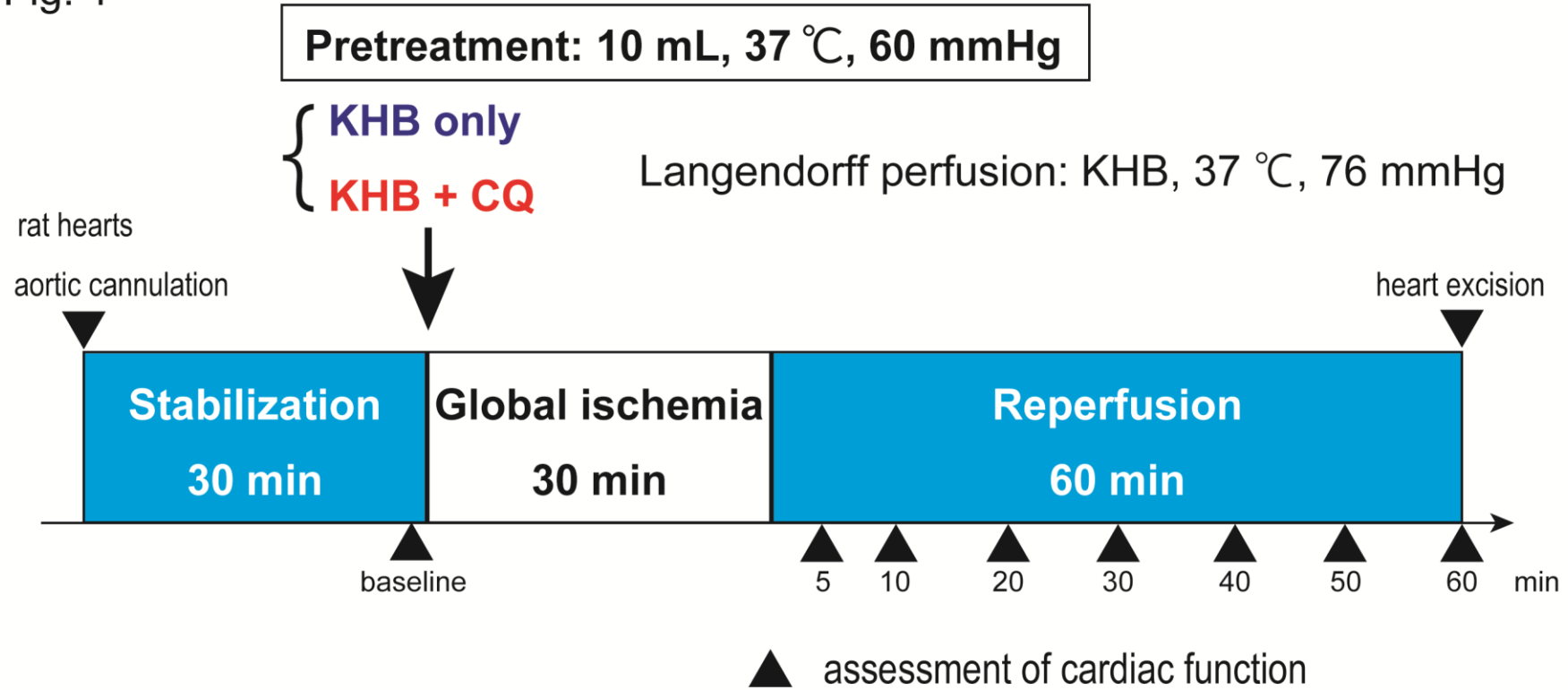


Fig. 2

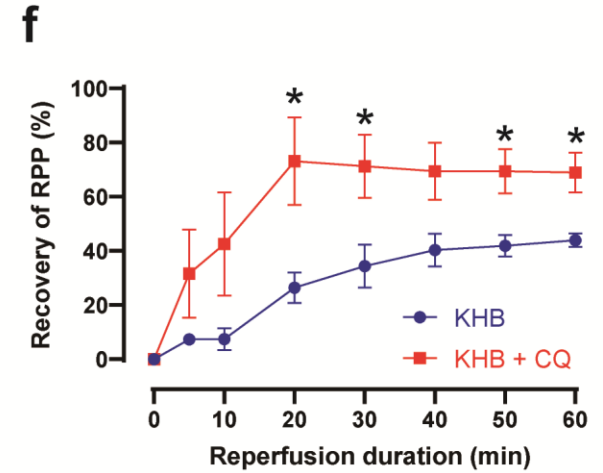
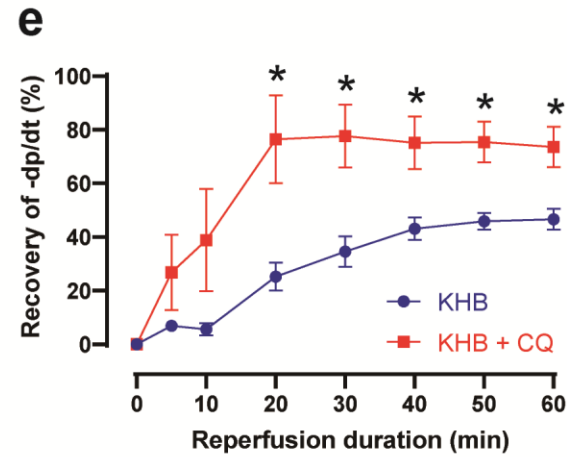
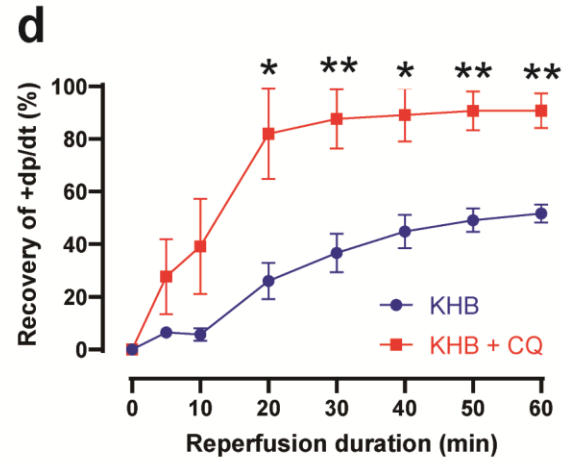
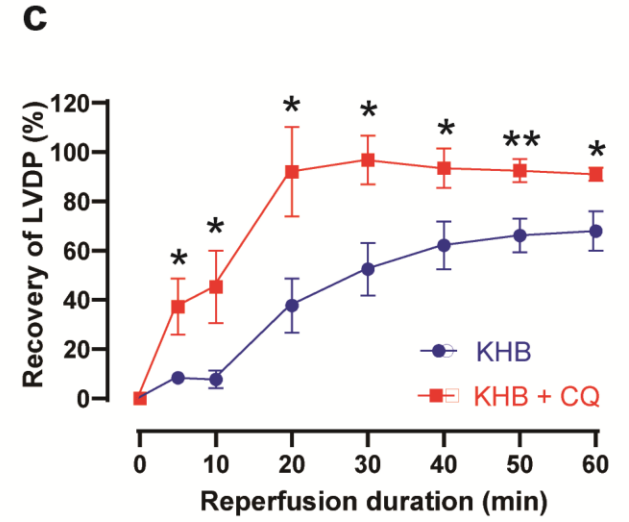
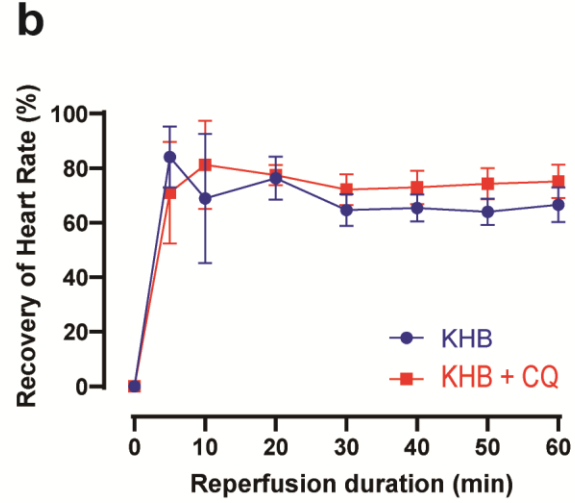
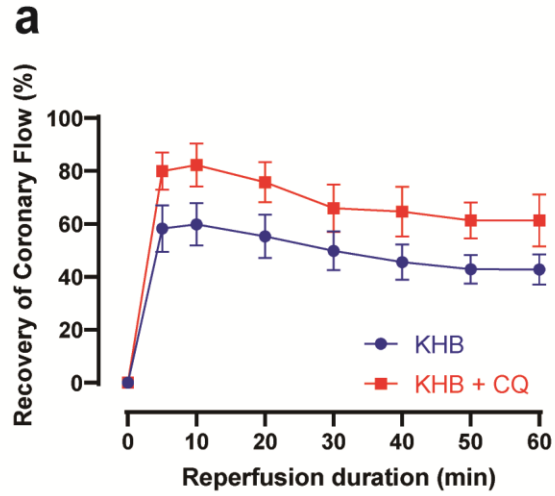
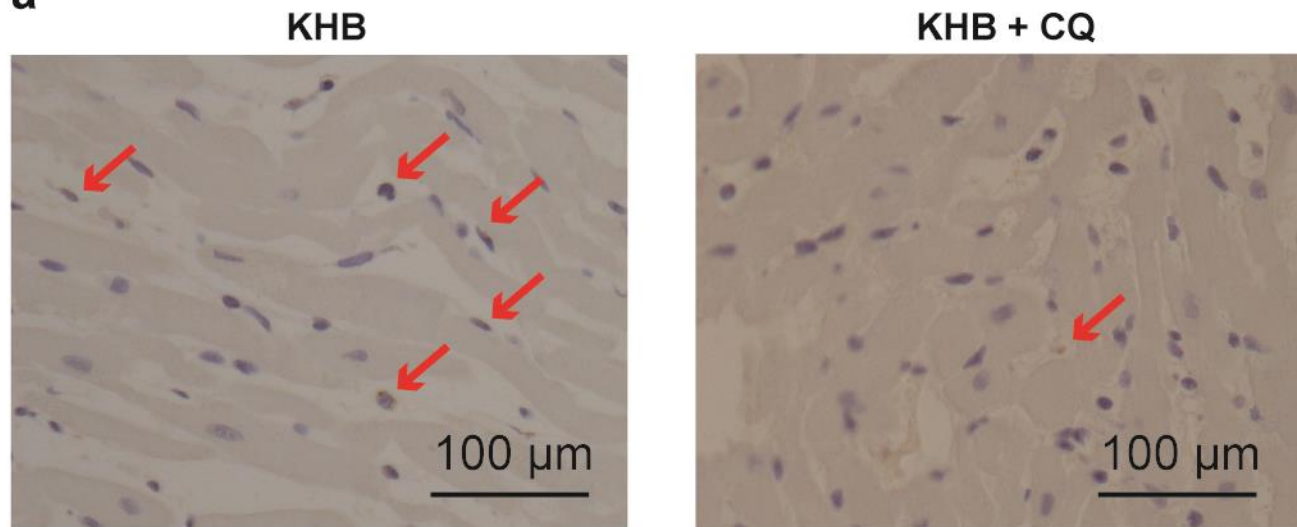


Fig. 3

a



b

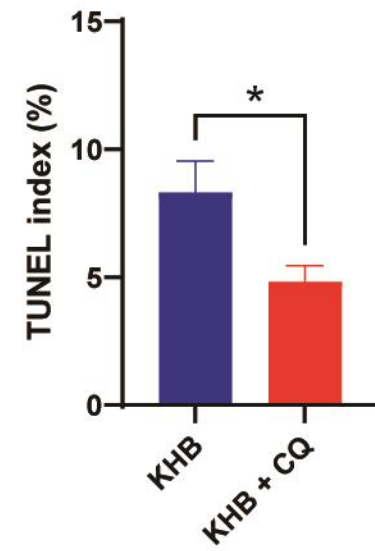
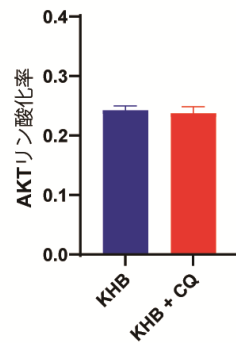
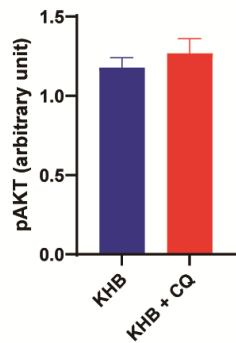
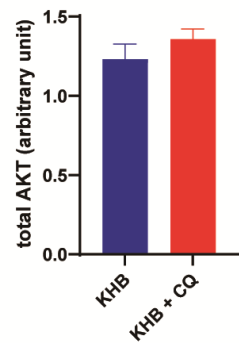
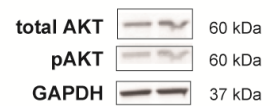


Fig. 4

a



b

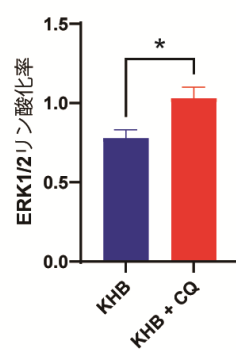
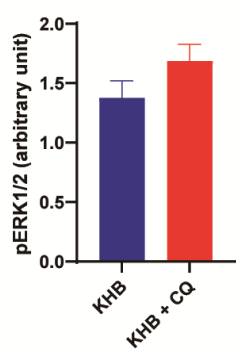
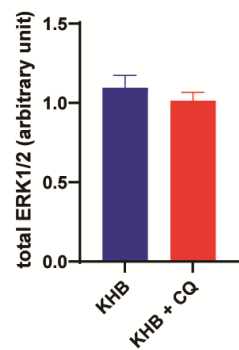
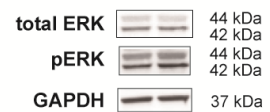


Fig. 5

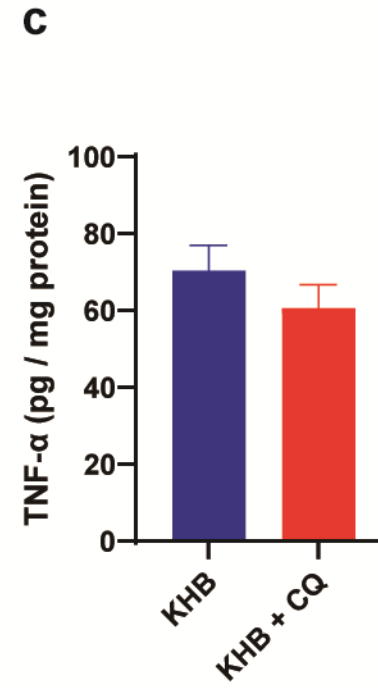
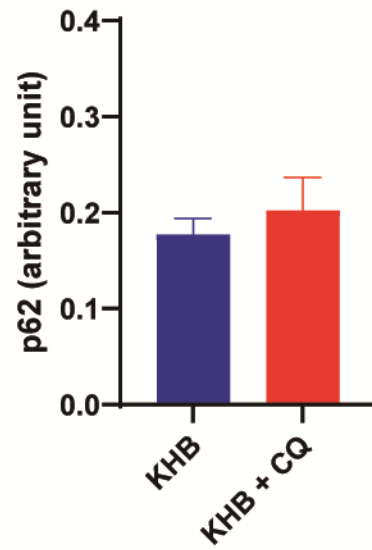
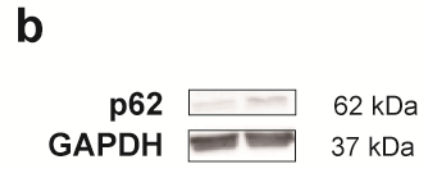
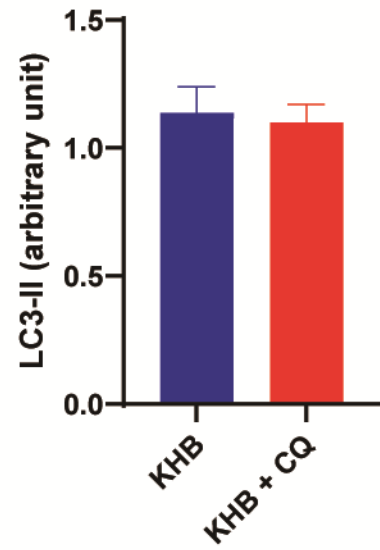
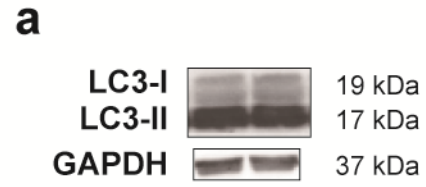
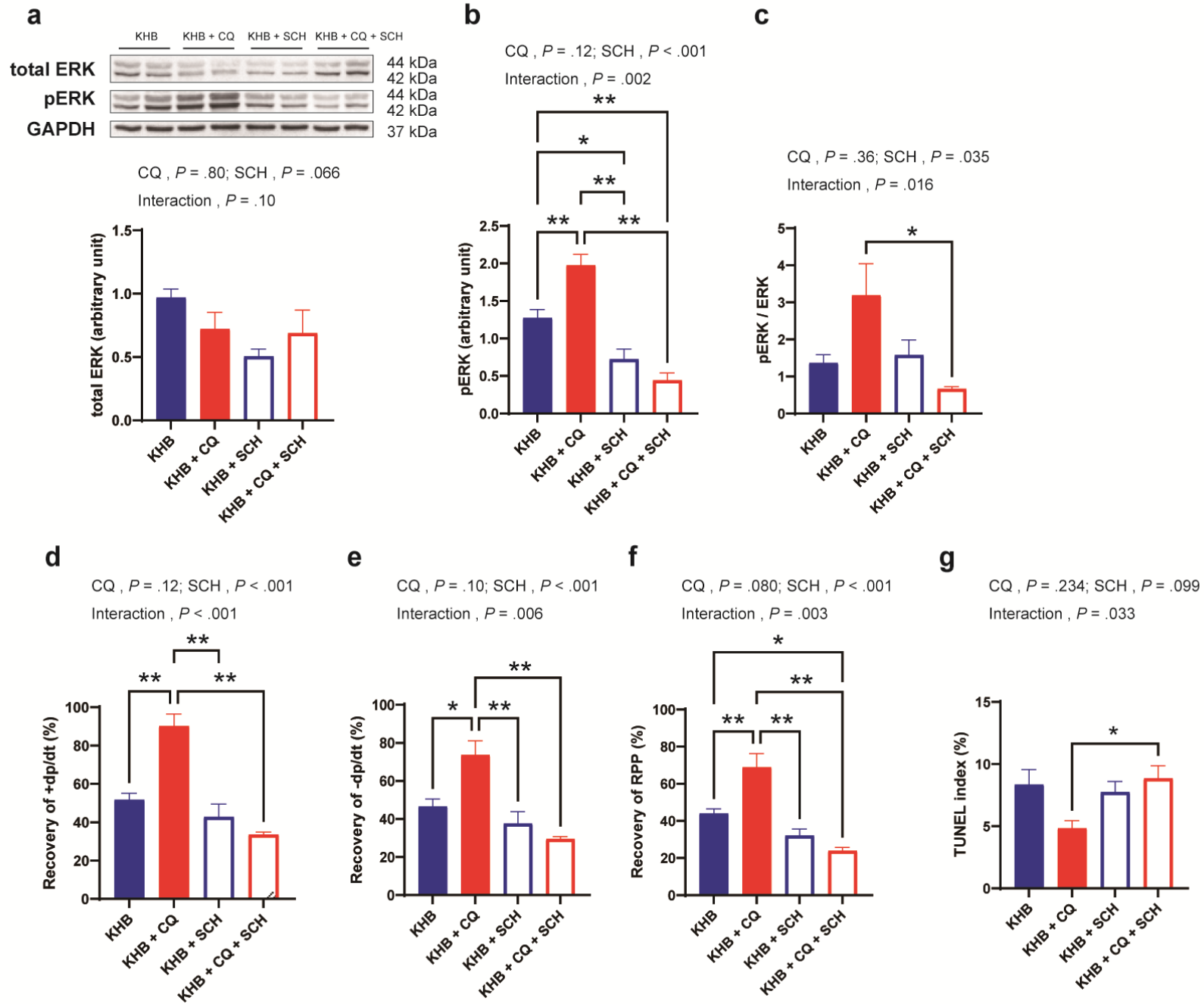


Fig. 6



Supplemental Fig. 1

