# Protective effects of trehalose preconditioning on cardiac and coronary endothelial function through eNOS signaling pathway in a rat model of ischemia-reperfusion injury

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## Abstract

Coronary endothelial dysfunction is a major cause of ischemia-reperfusion (I/R) injury. Trehalose, a natural disaccharide, has been reported to ameliorate endothelial dysfunction during aging by activating endothelial nitric oxide synthase (eNOS); however, its role in I/R injury is unknown. This study evaluated the effects of trehalose preconditioning on cardiac and coronary endothelial function after I/R. Langendorff-perfused rat hearts underwent 30 min of global ischemia followed by 80 min of reperfusion with or without trehalose preconditioning. Rate pressure product (RPP) and coronary flow (CF) were measured during reperfusion. Perivascular edema was assessed by hematoxylin and eosin staining. Myocardial oxidative stress and apoptosis were evaluated by immunohistochemistry and TUNEL staining, respectively. eNOS dimerization was determined by western blotting. An eNOS inhibitor was used to examine the role of eNOS. Trehalose preconditioning showed a higher recovery rate after I/R as indicated by high RPP (control vs. trehalose, 28 ± 6% vs. 46 ± 9%; P = 0.017, Cohen's $d$ = 2.3) and CF values (35 ± 10% vs. 55 ± 9%; P = 0.025, $d$ = 1.7). Furthermore, trehalose preconditioning reduced perivascular edema, myocardial oxidative stress, and apoptosis. The eNOS dimerization ratio was increased by trehalose (1.2 ± 0.2 vs. 1.6 ± 0.2; P = 0.023, $d$ = 2.1), which was associated with the recovery of RPP and CF. These effects of trehalose were abolished by the eNOS inhibitor. Trehalose preconditioning showed protective effects on cardiac and coronary endothelial function after I/R through the eNOS signaling pathway.

**Keywords**

Trehalose, Endothelial dysfunction, Endothelial nitric oxide synthase, Ischemia-reperfusion injury, Cardioprotection

## Introduction

Despite advances in surgical proficiency and medical technology, the rate of postoperative complications has increased in recent years with the increase in disease severity and operative complexity [1]. Since ischemia-reperfusion (I/R) injury has been recognized as a significant factor associated with mortality and morbidity during cardiac surgery, myocardial protection against I/R injury needs to be further improved [2, 3]. I/R injury is a complex process involving many mechanisms, including oxidative stress, inflammation, apoptosis, and autophagy [4]. Pharmacological preconditioning is one of the strategies employed to address the mechanisms leading to I/R injury, since it is known to ameliorate I/R injury due to administration of medication before ischemia [5, 6]. Although several medications for pharmacological preconditioning have been reported, the results of clinical research are unsatisfactory and they are yet to be routinely applied in cardiac surgery [7–9]. One reason for the failure of bench-to-bedside translation may be related to the fact that many studies have focused on cardiomyocytes and neglected other types of cells [10, 11]. The vascular endothelial cell is one such cell type that may play an important role in I/R injury. These cells not only serve as a protective barrier between cells, but also to contribute to the regulation of blood flow, inflammatory response, and myocardial function [12]. Therefore, we investigated whether the coronary endothelium could be a potential target for pharmacological preconditioning against I/R injury.

Trehalose (TRE), also known as an autophagy inducer, is a natural disaccharide found in several organisms. TRE plays a protective role in response to stress conditions such as heat, freezing, desiccation, dehydration, and oxidation [13]. Several studies have shown that TRE ameliorates endothelial dysfunction caused by $CdCl_2$ toxicity and aging by activating endothelial nitric oxide synthase (eNOS) [14, 15]. eNOS contributes to the production of nitric oxide (NO) which dilates the coronary arteries by relaxing the vascular smooth muscles [16]. The effect of TRE on coronary endothelial function after I/R is unknown and the role of eNOS remains to be clarified.

This study aimed to evaluate the cardioprotective effects of TRE against I/R injury in a model of isolated rat hearts and to elucidate the involvement of eNOS in TRE-mediated effect on cardiac and coronary endothelial function after I/R.

## Methods

### Animals

Male Sprague-Dawley rats (10 weeks, 290–340 g) were obtained from Sankyo Labo Service Corporation. The rats were maintained in standard animal care conditions (at 22 to 24 ℃, with 40% to 60% humidity, and a 12 h light-dark cycle), with commercial standard meals and city water.

### Preparation of Isolated Heart

Rats were anesthetized by intraperitoneal injection of sodium pentobarbital (150 mg/kg, Nacalai Tesque). To prevent blood coagulation, heparin (1000 IU/kg, Mochida Pharmaceutical Co., Ltd) was injected into the inferior vena cava. The hearts were then rapidly excised and the aorta was cannulated. The hearts were mounted in the Langendorff system (LaboSupport) and perfused with Krebs-Henseleit buffer (KHB) containing the following substances (mM, Sigma-Aldrich): NaCl 128, KCl 5.0, $MgSO_4$ 1.3, $KH_2PO_4$ 1.0, $CaCl_2$ 2.5, $NaHCO_3$ 15, and glucose 5.0 at pH 7.4. The buffer was equilibrated with 95% $O_2$ and 5% $CO_2$ at 37 ℃. The constant perfusion pressure was set at 76 mmHg and the chamber temperature was maintained at 37 ℃.

### Experimental Protocol

The experimental protocol is described in figure 1. All rat hearts were subjected to 20 min of perfusion with KHB for stabilization, 30 min of preconditioning, and 30 min of global ischemia followed by 80 min of reperfusion with KHB. The experimental protocol was based on the guidelines for experimental model of myocardial ischemia [17, 18]. The hearts were randomly assigned to the following two groups to measure the effects of TRE on cardiac function after I/R. In the control (CON) group (n = 4), hearts were perfused with only KHB; in the TRE group (n = 4), hearts were perfused with KHB containing 2% trehalose (HAYASHIBARA) during the preconditioning period. (Fig. 1a)

To evaluate whether TRE mediates cardioprotection through eNOS activation, the other eight hearts received 0.1 mM $N^G$-nitro-L-arginine methyl ester (L-NAME, an eNOS inhibitor; FUJIFILM Wako) during the preconditioning period and the entire reperfusion period. (Fig. 1b)

After reperfusion, the hearts were taken from the Langendorff system. The apex of the left ventricle was cut and then fixed in 3.5% neutral formalin, embedded in paraffin, and sectioned at 5 μm thickness. The other

tissues of the ventricle were immediately frozen in liquid $N_2$ and stored at −80 ℃ for different assays.

**Measurement of Cardiac Function**

A water-filled latex balloon connected to a pressure transducer was inserted into the left ventricle through the left atrium and the end-diastolic pressure of the left ventricle was adjusted to 5 mmHg. Pressure data were recorded using PowerLab, data acquisition device (ADInstruments) and analyzed using LabChart software (ADInstruments). Cardiac function was evaluated based on heart rate (HR), left ventricular developed pressure (LVDP), maximal increase and decrease velocity of left ventricular pressure (+dp/dt and −dp/dt), and rate pressure product (RPP = HR × LVDP). Coronary flow (CF) was measured based on timed collection of the coronary effluent using a measuring cylinder. Coronary vascular resistance was calculated using Ohm's law [18].

The inclusion criteria for the experimental animals were as follows: HR ≥ 180 beats/min, CF ≥ 8 ml/min, and LVDP ≥ 90 mmHg during the stabilization period. Baseline values of cardiac functional parameters were recorded at the end of the 20-min stabilization period. Recovery of each cardiac functional parameter during reperfusion was expressed as a percentage of the baseline value.

**Hematoxylin and Eosin Staining**

Myocardial perivascular edema was assessed by hematoxylin and eosin (H&E) staining. Perivascular edema is believed to be a sign of endothelial dysfunction after I/R injury [10, 19]. Three randomly chosen coronary vessels in each section were analyzed to measure edema and vascular areas using ImageJ software (NIH). The extent of perivascular edema was expressed as the ratio of the edema area to the vascular area [20].

**Immunohistochemistry**

To investigate the effect of TRE on I/R-induced oxidative stress, the expression of 4-hydroxynonenal (4-HNE), which reflects lipid peroxidation in myocardial tissue [21] was evaluated by immunohistochemistry. The sections were deparaffinized and treated with a target retrieval solution (Agilent) for antigen activation. After incubation in 3% $H_2O_2$, the sections were further incubated for 1 h with mouse anti-4 HNE antibody (R&D Systems) at room temperature, followed by 10-min incubation with anti-mouse secondary antibody (Agilent). The chromogenic reaction was visualized using diaminobenzidine (Agilent), and the nuclei were counterstained

with hematoxylin. The sections were analyzed using the ImageJ software (NIH). The percentage of the 4-HNE positive area as compared to the whole tissue area was calculated.

**TUNEL Staining**

Cell apoptosis is an important process that leads to cell death caused by myocardial I/R injury. The terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate nick end labeling (TUNEL) assay was performed to detect myocardial cell apoptosis [22]. The sections were deparaffinized and rehydrated in a concentration gradient of ethanol. The sections were permeabilized for 15 min in proteinase K buffer and incubated for 5 min in 3% $H_2O_2$ to inactivate endogenous peroxidase. Subsequently, the sections were incubated with TdT buffer (TaKaRa) for 70 min followed by incubation with anti-FITC horseradish peroxidase (HRP)-conjugated antibody solution (TaKaRa) for 30 min. The chromogenic reaction was visualized using diaminobenzidine (Agilent), and the nuclei were counterstained with hematoxylin. Ten randomly chosen frames in the sections were analyzed using the ImageJ software (NIH). The TUNEL index (%) was calculated by dividing the number of TUNEL positive cells by the total number of cells.

**Enzyme-linked Immunosorbent Assay**

To investigate the effect of TRE on I/R induced inflammatory response, the level of tumor necrosis factor-α (TNF-α) were detected by enzyme-linked immunosorbent assay (ELISA). The frozen myocardial tissue samples were homogenized in the sample diluent buffer. The homogenates were centrifuged at 16000 × g for 10 min and the supernatants were assayed using an ELISA kit (BioVision Inc.) according to the manufacturer's instructions. Values are expressed as pg of TNF-α per mg of protein.

**Western Blotting**

eNOS dimerization has been established to be important for its normal function [19]. The dimerization of eNOS was determined by the dimer monomer ratio. eNOS phosphorylation has been reported to increase through Akt, extracellular signal-regulated kinase (ERK), and AMP-activated protein kinase α (AMPKα) [20,21]. To evaluate autophagy activation, the expression of two major autophagy markers, microtubule-associated protein 1 light chain 3 (LC3) and p62/Sequestosome 1 (p62) was evaluated. LC3-II serves as a marker for autophagosome formation, and p62 is also commonly used as a marker for autophagic degradation [23]. The

expression of caspase-3 was evaluated as a marker for apoptosis.

The frozen myocardial tissue samples were ground using a mortar and pestle and subsequently placed in a tissue grinder with lysis buffer. The lysates were centrifuged at 16000 × g for 10 min at 4 ℃, and the supernatants were collected. Protein concentration was determined by the Bradford protein assay. A semi-dry western blot apparatus (Mini-PROTEAN Tetra Cell, BIO-RAD) was used. Protein samples (25 μg) were separated on a 12% gel by SDS polyacrylamide gel electrophoresis (Mini-PROTEANTGXTM, BIO-RAD) and blotted onto PVDF membranes (Millipore). The membranes were then blocked with an ECL blocking agent (Cytiva) in Tris-buffered saline containing 0.1% Tween 20 at room temperature for 1 h. After blocking, the membranes were incubated with rabbit primary antibodies against caspase-3 (1:1000, Cell Signaling [CS]), eNOS (1:1000, CS), p-eNOS [Ser1177] (1:1000, CS), Akt (1:1500, CS), p-Akt [Ser473] (1:1500, CS), ERK1/2 (1:1200, CS), p-ERK1/2 [Thr202/Tyr204] (1:2000, CS), AMPK (1:1000, CS), p-AMPK [Thr172] (1:1000, CS), LC3 (1:500, Abcam), p62 (1:10000, CS), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1:1000, CS) at 4 ℃ overnight. Immune complexes were detected using HRP-conjugated secondary antibodies (1:2000, anti-rabbit IgG, CS) at room temperature for 1 h. Protein bands were detected and semi-quantified using the chemiluminescence system (ECL Plus, Cytiva) and JustTLC analysis software (SWEDAY), respectively. Protein expression was normalized to that of GAPDH, used as a loading control.

To evaluate the eNOS dimer, we performed 'low-temperature SDS-PAGE' as described previously [24]. Briefly, samples were not boiled, and were mixed with the loading buffer without β-mercaptoethanol and separated on a 4%–20% gel by SDS-polyacrylamide gel electrophoresis (BIO-RAD) at 4 ℃.

**Statistical Analysis**

All data are expressed as the mean ± standard error of the mean (SEM). The calculated sample size was n = 4 for detecting a 20% mean difference in RPP recovery (power, 80%; $\alpha < 0.05$). Student's t-test was performed for comparison between two groups. To analyze differences between the four groups, one-way ANOVA with post-hoc Tukey's test was employed. Correlations between the parameters were analyzed using Pearson's correlation test. Comparisons of hemodynamic parameters during preconditioning among groups or time points was analyzed using two-way repeated ANOVA. Statistical significance was set at $P < 0.05$. Additionally, we calculated the effect size (Cohen's *d*) to evaluate the differences. In general, the effect size is very large if $d \geq 1.3$ [25]. Statistical analyses were performed using the GraphPad Prism software, version 9 (GraphPad

Software).

## Results

### Cardiac Function after I/R

Baseline hemodynamic parameters in the Langendorff-perfused hearts are shown in table 1. There were no significant differences in the hemodynamic parameters between the CON and TRE groups, even during the preconditioning period. Furthermore, there was no statistical interaction between groups and time points.

The recovery rate in cardiac function (HR, LVDP, +dp/dt, −dp/dt, RPP) after I/R was compared between the CON and TRE groups (Fig. 2). In the TRE group, the recovery of LVDP (CON, 45 ± 6% vs. TRE, 77 ± 13%; P = 0.004, $d$ = 3.3), +dp/dt (CON, 54 ± 4% vs. TRE, 74 ± 8%; P = 0.004, $d$ = 3.2), −dp/dt (CON, 44 ± 21% vs. TRE, 81 ± 8%; P = 0.015, $d$ = 2.4), and RPP (CON, 28 ± 6% vs. TRE, 46 ± 9%; P = 0.017, $d$ = 2.3) was higher after 80 min of reperfusion than in the CON group. HR did not differ between the two groups, except at 10 min after reperfusion due to arrhythmia. The recovery of CF was higher in the TRE group from the beginning to the end of reperfusion than in the CON group (CON, 35 ± 10% vs. TRE, 55 ± 9%; P = 0.025, $d$ = 1.7), indicating a lower coronary vascular resistance in the TRE group.

### Oxidative Stress, Inflammation, and Apoptosis in Myocardial Tissue

Oxidative stress, inflammation, and apoptosis in myocardial tissue after I/R were compared between the CON and TRE groups (Fig. 3). Immunohistochemical analysis revealed a smaller 4-HNE positive area in the TRE group than in the CON group (CON, 12 ± 3% vs. TRE, 6 ± 2%; P = 0.011, $d$ = 3.0; Fig. 3a and 3b), suggesting a decrease in myocardial oxidative stress upon TRE treatment. There was no difference in the levels of TNF-α between the two groups (Fig. 3c). The TUNEL index was lower in the TRE group than in the CON group after I/R (CON, 57 ± 7% vs. TRE, 21 ± 11%; P = 0.004, $d$ = 3.8; Fig. 3d and 3e). The expression of cleaved caspase-3 was also lower in the TRE group than in the CON group (CON, 0.11 ± 0.03 vs. TRE, 0.06 ± 0.02 arbitrary unit; P = 0.045, $d$ = 1.8; Fig. 3f), suggesting a decrease in myocardial apoptosis upon TRE treatment.

### eNOS Activity in Myocardial Tissue

eNOS phosphorylation and dimerization after I/R were compared between the CON and TRE groups (Fig. 4).

There was no difference in the phosphorylation of eNOS at serine 1177 between the two groups (Fig. 4a). On the other hand, eNOS dimerization (dimer monomer ratio) was higher in the TRE group than in the CON group (CON, 1.2 ± 0.2 vs. TRE, 1.6 ± 0.2 arbitrary unit; P = 0.023, *d* = 2.1; Fig. 4b), suggesting that eNOS was activated in the TRE group.

**Expression of Signaling Proteins Related to eNOS Phosphorylation**

The expression level of signaling proteins related to eNOS phosphorylation after I/R were compared between the CON and TRE groups (Fig. 5). There was no difference in the expression of total Akt between the two groups. On the other hand, the levels of p-Akt were lower in the TRE group than in the CON group (CON, 0.6 ± 0.1 vs. TRE, 0.4 ± 0.1 arbitrary unit; P = 0.039, *d* = 1.9; Fig. 5a). There was no difference in the expression of either total ERK1/2 or p-ERK1/2 between the two groups (Fig. 5b). There was no difference in the expression of either total AMPKα or p-AMPKα between the two groups (Fig. 5c).

**Autophagy Markers**

The expression levels of autophagy markers, LC3-II and p62, after I/R were compared between the CON and TRE groups (Fig. 6). In the TRE group, the expression levels of LC3-II were higher than that in the CON group (CON, 0.6 ± 0.1 vs. TRE, 0.8 ± 0.1 arbitrary unit; P = 0.032, *d* = 2.0), indicating increased number of autophagosomes in the TRE group. The expression levels of p62 did not differ between the two groups (Fig. 6a). The expression of LC3-II was strongly positively correlated with eNOS dimerization (Fig. 6b). Furthermore, the expression of LC3-II was positively correlated with cardiac functional parameters (LVDP, +dp/dt, −dp/dt, and RPP) and CF, a surrogate marker of coronary endothelial function (Fig. 6c).

**Effects of an eNOS Inhibitor on Cardioprotection Induced by Trehalose**

The effects of L-NAME, an eNOS inhibitor, on coronary endothelial function are shown in figure 7. Representative images of H&E staining of the perivascular area are shown in figure 7a. The hearts in the TRE group had less perivascular edema after I/R than that in the CON group. In the presence of L-NAME, TRE-induced reduction of perivascular edema (Fig. 7a and 7b) and coronary vascular resistance (Fig. 7c) were abolished.

The effects of L-NAME on the recovery of RPP, myocardial 4-HNE positive area, and TUNEL index

were evaluated (Fig. 8). After reperfusion, the recovery of RPP, induced by TRE, was reduced to the level of the CON group in the presence of L-NAME (Fig. 8a). Moreover, other cardiac functional parameters (LVDP, +dp/dt, −dp/dt) showed a pattern similar to RPP. In addition, the effect of TRE in reducing the myocardial 4-HNE positive area was abolished in the presence of L-NAME (Fig. 8b). In contrast, L-NAME administration did not change the effect of TRE on the TUNEL index (Fig. 8c).

## Discussion

In the present study, we demonstrated that TRE preconditioning attenuated cardiac and coronary endothelial dysfunction after I/R and the effects of TRE were dependent on eNOS signaling.

eNOS is a homodimeric enzyme that is important for endothelial function and is highly expressed in coronary endothelial cells [26]. Under physiological conditions, eNOS synthesizes NO and l-citrulline from l-arginine and oxygen. The activity of eNOS depends on many factors, including multiple cofactors and the phosphorylation of multiple amino acids. Furthermore, it has been established that eNOS dimerization is necessary for normal eNOS function [16, 27]. In its monomeric form, eNOS is unable to catalyze the conversion of l-arginine to l-citrulline for NO production.

Several studies have provided evidence that limited eNOS dimerization is associated with many cardiovascular diseases, including heart failure, atrial fibrillation, hypertension, atherosclerosis, and I/R [28]. During I/R, eNOS dimerization is an important factor for endothelium-dependent coronary vasoreactivity [29]. Several preconditioning methods (e.g., exercise training and inhalational anesthesia) have been reported to ameliorate endothelial dysfunction through eNOS dimerization [30, 31]. In the present study, we demonstrated that TRE increased eNOS dimerization, reduced coronary vascular resistance and perivascular edema after I/R compared to the CON group. Since pharmacological inhibition of eNOS abolished the effects of TRE, the eNOS signaling pathway plays an important role in the mechanism of TRE preconditioning.

In the present study, the mechanism of eNOS dimerization mediated by TRE is unclear. Autophagy has been recently proposed to be involved in eNOS dimerization [32]. TRE has been reported to promote autophagy by decreasing the phosphorylation of Akt at Ser473 [33–35]. Consistent with previous studies, we found that TRE increased the expression of the autophagosome marker LC3-II, and decreased p-Akt levels. Furthermore, LC3-II expression was strongly positively correlated with eNOS dimerization. On the contrary, TRE did not

alter the expression of the autophagic degradation marker, p62. Since TRE has been reported to increase p62 expression in an autophagy-independent manner [36], p62 may not be an appropriate marker of autophagic activity in this model of TRE preconditioning. Further studies using autophagy inhibitors are needed to investigate the involvement of autophagy in TRE preconditioning.

Here, we demonstrated the protective effect of TRE against I/R-induced apoptosis independent of eNOS signaling and lipid peroxidation. Apoptosis is the process of programmed cell death that is generally induced upon oxidative stress. Several studies have shown that TRE prevents apoptosis and lipid peroxidation caused by I/R in the kidneys and myocardial cells [37, 38]. However, the causal relationship between lipid peroxidation and apoptosis is unclear in these previous studies. The mechanism of the anti-apoptotic effect of TRE is still unknown, and further studies are necessary.

The beneficial effects of TRE on coronary endothelial function were associated with the preservation of cardiac function. Possible reasons for this could be promotion of microcirculation, the reduction of capillary permeability, and the increase in paracrine signaling of endothelial cells in the myocardium [39–41] . Thus, the coronary endothelium may be an important target for TRE preconditioning in the setting of I/R.

TRE is a natural compound found in certain foods and is not synthesized by the human body. TRE ingested from food is enzymatically hydrolyzed into two D-glucose molecules in the small intestine. However, no apparent side effects have been reported in humans [42]. In addition, TRE has been used as a preservation solution for lung transplants [43]. TRE may have translational potential as a cardioplegia solution in cardiac surgery and as a preservation solution for heart transplants.

There are several limitations in this study. First, the method of TRE administration was selected based on our previous study; however, the most appropriate concentration and duration of TRE preconditioning have not been investigated. Further studies in this regard will improve the efficacy of cardioprotection by TRE. Second, examining the bioavailability of NO and generation of reactive nitrogen species will provide more evidence regarding the role of eNOS in TRE preconditioning.

## Conclusions

TRE preconditioning ameliorates I/R injury in rat hearts. eNOS signaling plays an important role in the preservation of cardiac and coronary endothelial functions mediated by TRE after I/R. In cardiac surgery,

including transplantation, TRE preconditioning could be a potential strategy for myocardial protection.

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## Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

## Author Contributions

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

## Data Availability

The datasets generated during the current study will be shared on reasonable request to the corresponding author.

## Ethical approval

All applicable international and national guidelines for the care and use of animals were followed. And all experimental procedures were conducted according to the Hokkaido university manual for implementing animal experimentation. No human studies were carried out by the authors for this article.

## Figure legends

**Fig. 1** Experimental protocol: (a) Effects of trehalose on cardiac function after ischemia-reperfusion; (b) Role of endothelial nitric oxide synthase in the cardioprotective effect produced by trehalose. n = 4 for each group. CON, control; TRE, trehalose; L-NAME, $N^G$-nitro-L-arginine methyl ester

**Fig. 2** Cardiac functional parameters after ischemia-reperfusion; Recovery of each parameter was expressed as a percentage of baseline values during reperfusion: (a) HR; (b) CF; (c) LVDP; (d) +dp/dt; (e) −dp/dt; (f) RPP. Data are presented as the mean ± SEM. In each group, n = 4. *P < 0.05 compared with the control group at the same duration. Significance was calculated using Student's t-test. CF, coronary flow; CON, control; HR, heart rate; LVDP, left ventricular developed pressure; RPP, rate pressure product; TRE, trehalose

**Fig. 3** Myocardial oxidative stress, inflammation, and apoptosis after ischemia-reperfusion: (a) Representative pictures of anti 4-HNE immunostaining. Scale bar, 200 μm; (b) Quantitative analyses of the 4-HNE positive area; (c) Level of TNF-α; (d) Representative pictures of TUNEL staining. Scale bar, 20 μm. The arrows indicate TUNEL-positive cells; (e) TUNEL index; (f) Representative bands and corresponding analysis of cleaved caspase-3. Values are presented as the mean ± SEM. In each group, n = 4. *P < 0.05, **P < 0.01. Significance was calculated using Student's t-test. a.u., arbitrary unit; CON, control; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; 4-HNE, 4-hydroxynonenal; TNF-α, tumor necrosis factor-α; TRE, trehalose; TUNEL, terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling

**Fig. 4** Phosphorylation and dimerization of eNOS in the myocardium: (a) Representative bands and corresponding analysis of phosphorylated eNOS (Ser1177); (b) Representative bands and corresponding analysis of eNOS dimerization. Values are expressed as the mean ± SEM. In each group, n = 4. *P < 0.05. Significance was calculated using Student's t-test. a.u., arbitrary unit; CON, control; eNOS, endothelial nitric oxide synthase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; p-eNOS, phosphorylated eNOS; TRE, trehalose

**Fig. 5** Signaling proteins related to eNOS phosphorylation: (a) Representative bands and corresponding analysis of Akt and p-Akt (Ser473); (b) ERK and p-ERK (Thr202/Tyr204); (c) AMPK and p-AMPK (Thr172). Values are expressed as the mean ± SEM. In each group, n = 4. *P < 0.05. Significance was calculated using Student's t-test. a.u., arbitrary unit; AMPK, AMP-activated protein kinase; CON, control; ERK, extracellular signal-regulated kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; TRE, trehalose

**Fig. 6** Autophagy markers in the myocardium: (a) Representative bands and corresponding analysis of LC3-II and p62; (b) Correlation between the expression of LC3-II and eNOS dimerization; (c) Correlations between the expression of LC3-II and cardiac functional parameters. The r indicates the correlation coefficient. Values are expressed as the mean ± SEM. In each group, n = 4. *P < 0.05. Significance was calculated using Student's t-test and Pearson's correlation test. a.u., arbitrary unit; CF, coronary flow; CON, control; eNOS, endothelial nitric oxide synthase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HR, heart rate; LC3, microtubule-associated protein 1 light chain 3; LVDP, left ventricular developed pressure; RPP, rate pressure product; TRE, trehalose

**Fig. 7** Effects of an eNOS inhibitor on coronary endothelial function: (a) Representative pictures of hematoxylin and eosin staining. Scale bar, 20 μm. The arrows indicate perivascular edema; (b) Quantitative analyses of perivascular edema after I/R; (c) Coronary vascular resistance expressed as percentages of baseline during reperfusion. Values are expressed as the mean ± SEM. In each group, n = 4. **P < 0.01; $^{\#}$P < 0.05 vs. CON; $^{\$}$P < 0.05 vs. L-NAME; $^{\dagger}$P < 0.05 vs. TRE + L-NAME. Significance was calculated using one-way ANOVA with Tukey's post hoc test. CON, control; eNOS, endothelial nitric oxide synthase; I/R, ischemia-reperfusion; L-NAME, $N^G$-nitro-L-arginine methyl ester; TRE, trehalose

**Fig. 8** Effects of an eNOS inhibitor on cardiac function, myocardial oxidative stress, and apoptosis: (a) Recovery of RPP at the end of the reperfusion period; (b) Quantitative analyses of 4-HNE positive area; (c) TUNEL index. Values are expressed as the mean ± SEM. In each group, n = 4. *P < 0.05, **P < 0.01. Significance was calculated using one-way ANOVA with post hoc Tukey's test. CON, control; eNOS, endothelial nitric oxide synthase; 4-HNE, 4-hydroxynonenal; L-NAME, $N^G$-nitro-L-arginine methyl ester; RPP,

rate pressure product; TRE, trehalose; TUNEL, terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling

**Table1.** Baseline Hemodynamics in the Langendorff-perfused Hearts

| | | | Preconditioning | | |
|---|---|---|---|---|---|
| | | Baseline | 10 min | 20 min | 30 min |
| HR (beats/min) | CON | 212 ± 9 | 199 ± 5 | 190 ± 6 | 189 ± 6 |
| | TRE | 225 ± 8 | 206 ± 7 | 189 ± 5 | 175 ± 6 |
| CF (ml/min) | CON | 13 ± 2 | 11 ± 2 | 10 ± 2 | 10 ± 2 |
| | TRE | 12 ± 2 | 12 ± 2 | 11 ± 2 | 11 ± 2 |
| LVDP (mmHg) | CON | 108 ± 5 | 110 ± 6 | 99 ± 9 | 102 ± 8 |
| | TRE | 105 ± 6 | 109 ± 6 | 106 ± 3 | 98 ± 3 |
| +dp/dt ($\times 10^3$ mmHg/s) | CON | 2.9 ± 0.3 | 2.9 ± 0.2 | 2.7 ± 0.1 | 2.7 ± 0.2 |
| | TRE | 2.8 ± 0.3 | 3.3 ± 0.2 | 3.1 ± 0.2 | 2.8 ± 0.2 |
| -dp/dt ($\times 10^3$ mmHg/s) | CON | 1.5 ± 0.1 | 1.4 ± 0.1 | 1.3 ± 0.2 | 1.3 ± 0.2 |
| | TRE | 1.3 ± 0.1 | 1.3 ± 0.1 | 1.3 ± 0.1 | 1.2 ± 0.1 |
| RPP ($\times 10^4$ mmHg・beats/min) | CON | 2.3 ± 0.1 | 2.3 ± 0.1 | 1.9 ± 0.2 | 1.9 ± 0.1 |
| | TRE | 2.4 ± 0.2 | 2.2 ± 0.2 | 2.0 ± 0.1 | 1.7 ± 0.1 |

Data are presented as the means ± SEM. In each group, n = 4. Significance was calculated using two-way repeated measures ANOVA.

There were no significant differences in baseline hemodynamics between two groups. HR, heart rate; CF, coronary flow; LVDP, left ventricular developed pressure; +dp/dt, maximal rate of pressure rise; -dp/dt, maximal rate of pressure decrease; RPP, rate pressure product.

Fig. 1

a


CON
(n = 4)
Stabilization
Preconditioning
Ischemia
Reperfusion
trehalose
TRE
(n = 4)
Stabilization
Preconditioning
Ischemia
Reperfusion
20 min
30 min
30 min
80 min
Western blot analyses
Histopathological analyses

b

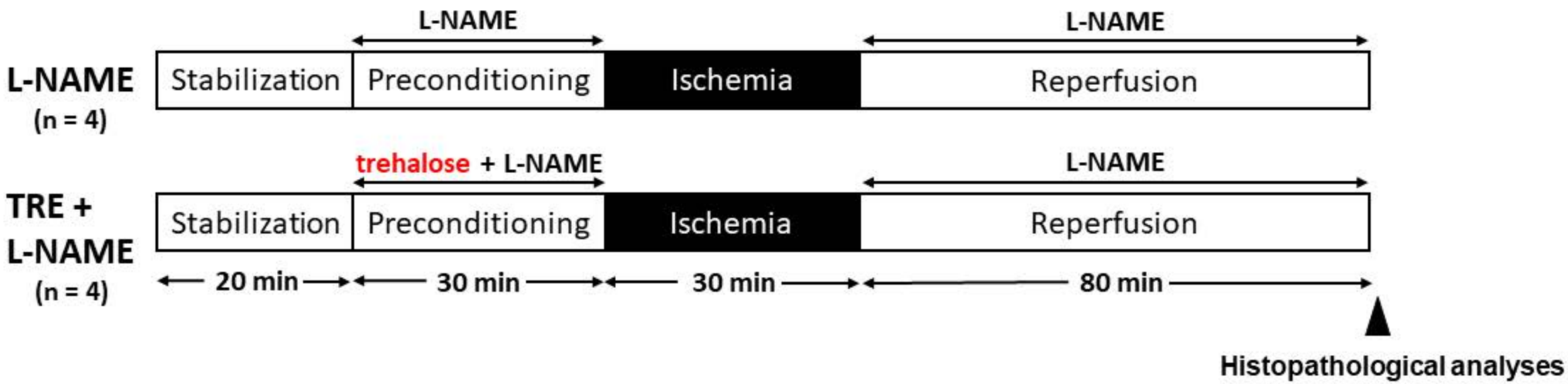
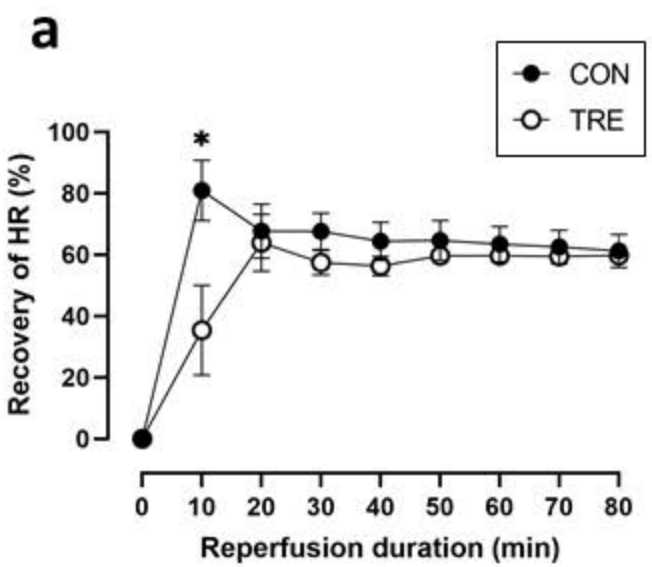
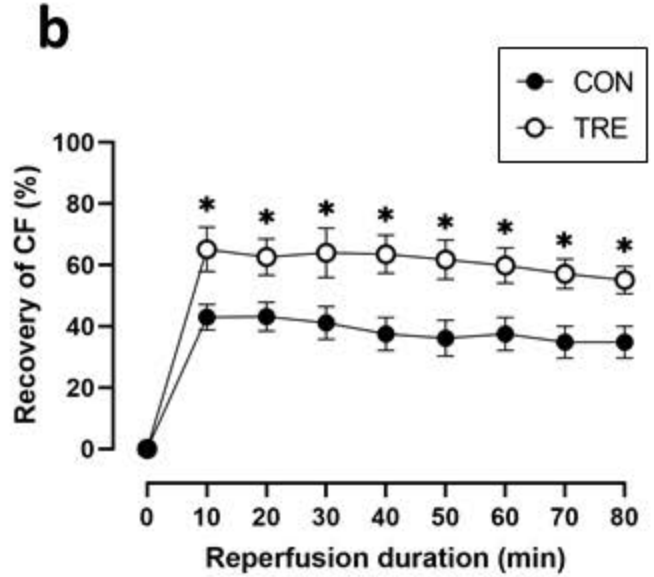
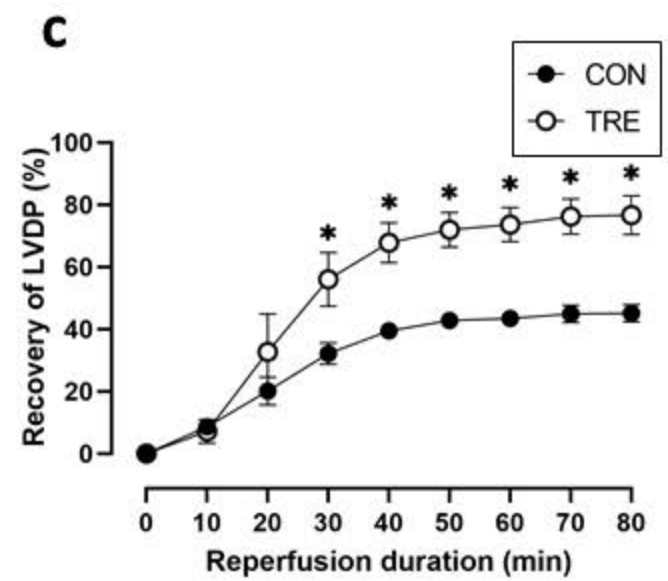
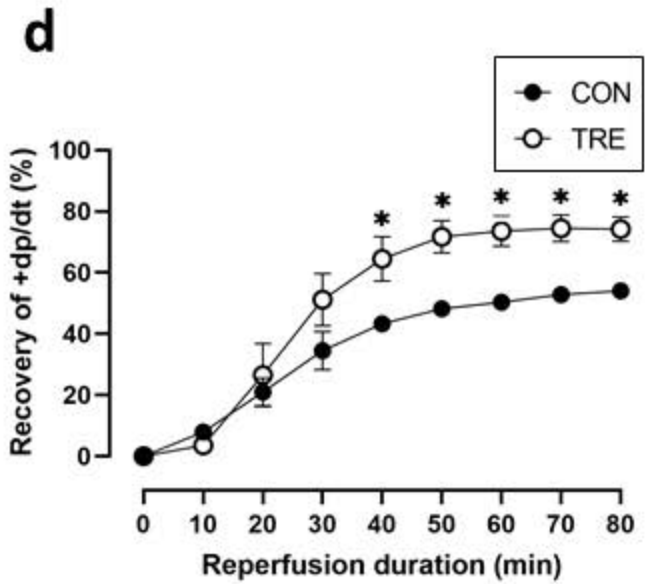

L-NAME
L-NAME
L-NAME
(n = 4)
Stabilization
Preconditioning
Ischemia
Reperfusion
trehalose + L-NAME
L-NAME
TRE +
L-NAME
(n = 4)
Stabilization
Preconditioning
Ischemia
Reperfusion
20 min
30 min
30 min
80 min
Histopathological analyses

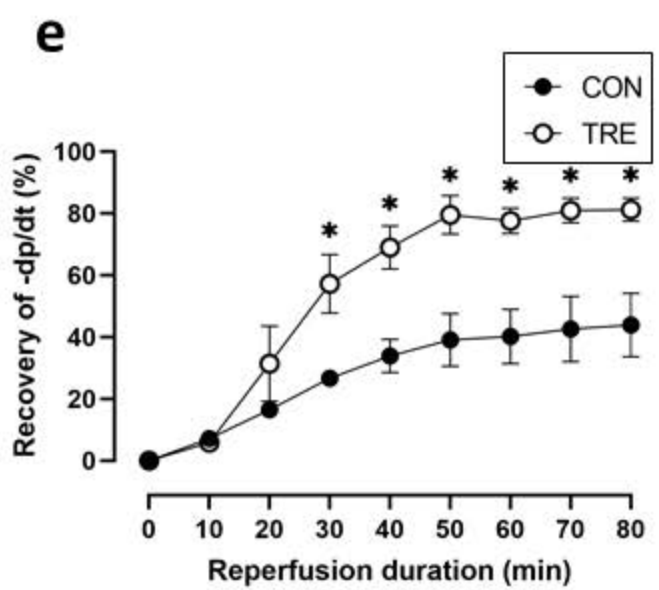
Fig. 2


a
CON
TRE
Recovery of HR (%)
Reperfusion duration (min)


b
CON
TRE
Recovery of CF (%)
Reperfusion duration (min)


c
CON
TRE
Recovery of LVDP (%)
Reperfusion duration (min)


d
CON
TRE
Recovery of +dp/dt (%)
Reperfusion duration (min)


e
CON
TRE
Recovery of -dp/dt (%)
Reperfusion duration (min)

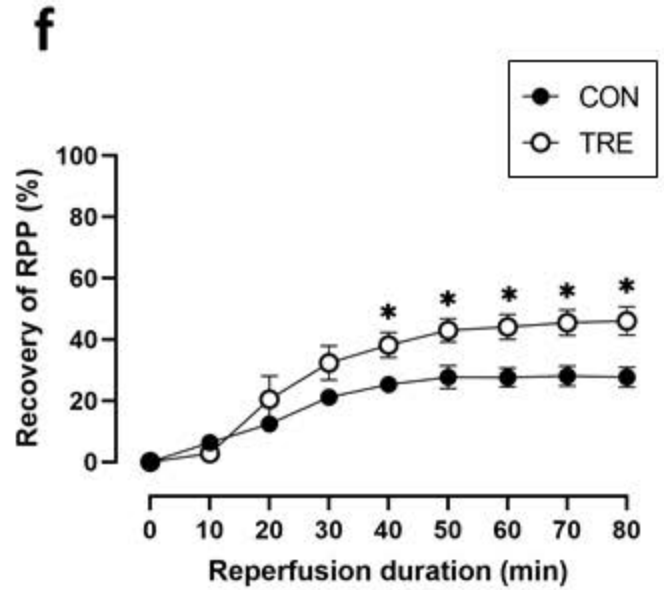

f
CON
TRE
Recovery of RPP (%)
Reperfusion duration (min)

Fig. 3

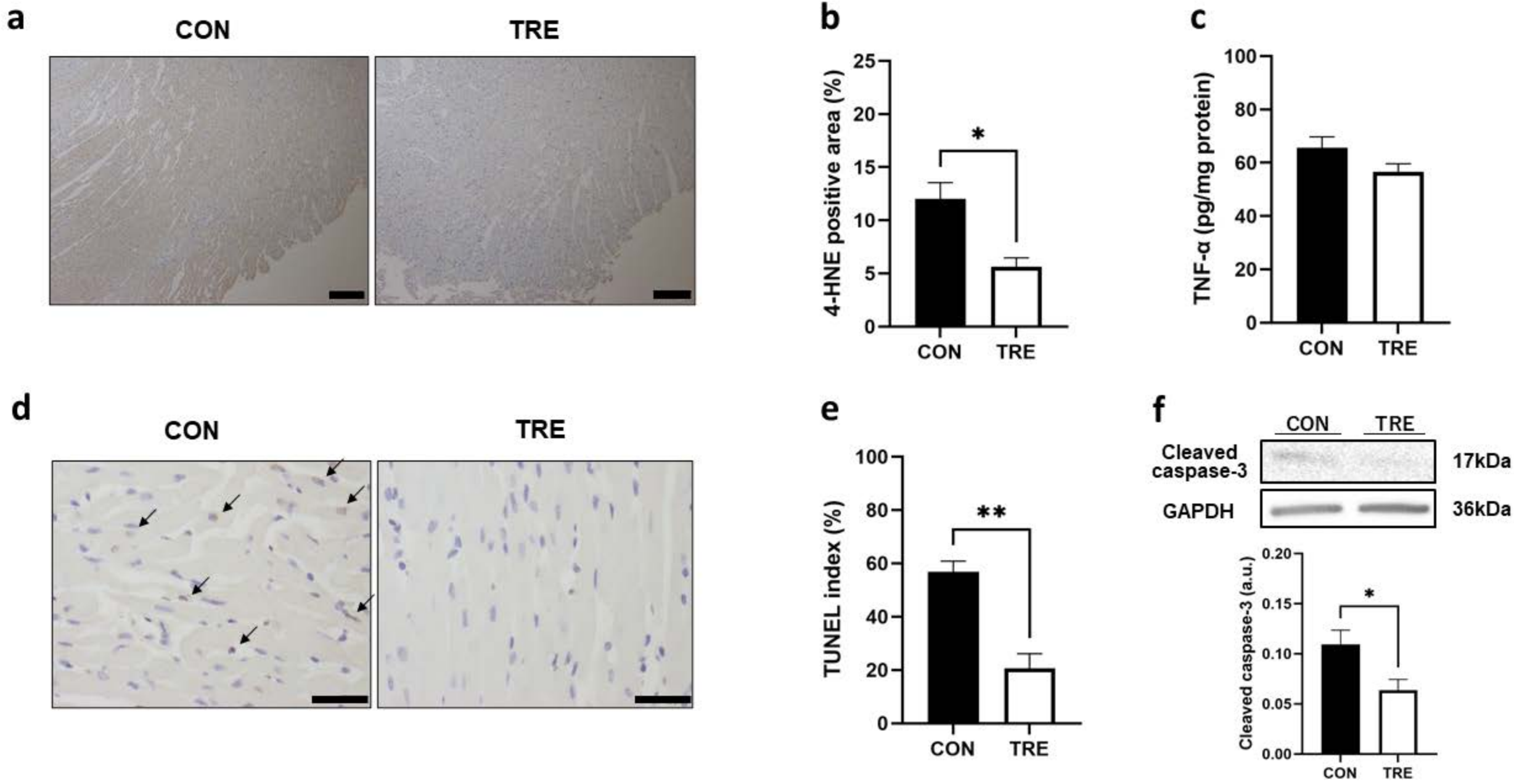

a
CON
TRE
b
4-HNE positive area (%)
25
20
15
10
5
0
*
CON
TRE
c
TNF-α (pg/mg protein)
100
80
60
40
20
0
CON
TRE
d
CON
TRE
e
TUNEL index (%)
100
80
60
40
20
0
**
CON
TRE
f
CON
TRE
Cleaved caspase-3
17kDa
GAPDH
36kDa
Cleaved caspase-3 (a.u.)
0.20
0.15
0.10
0.05
0.00
*
CON
TRE

Fig. 4

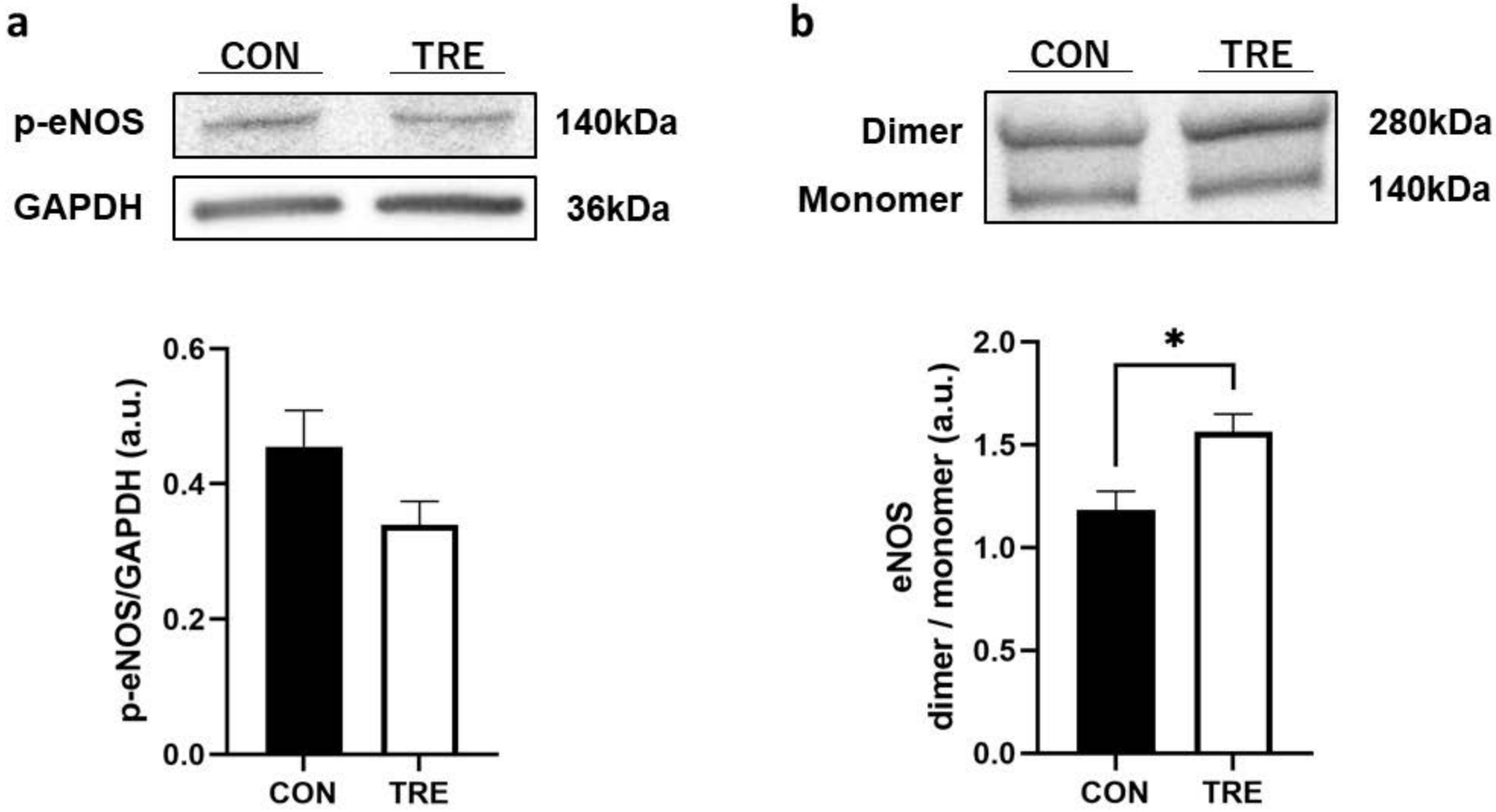
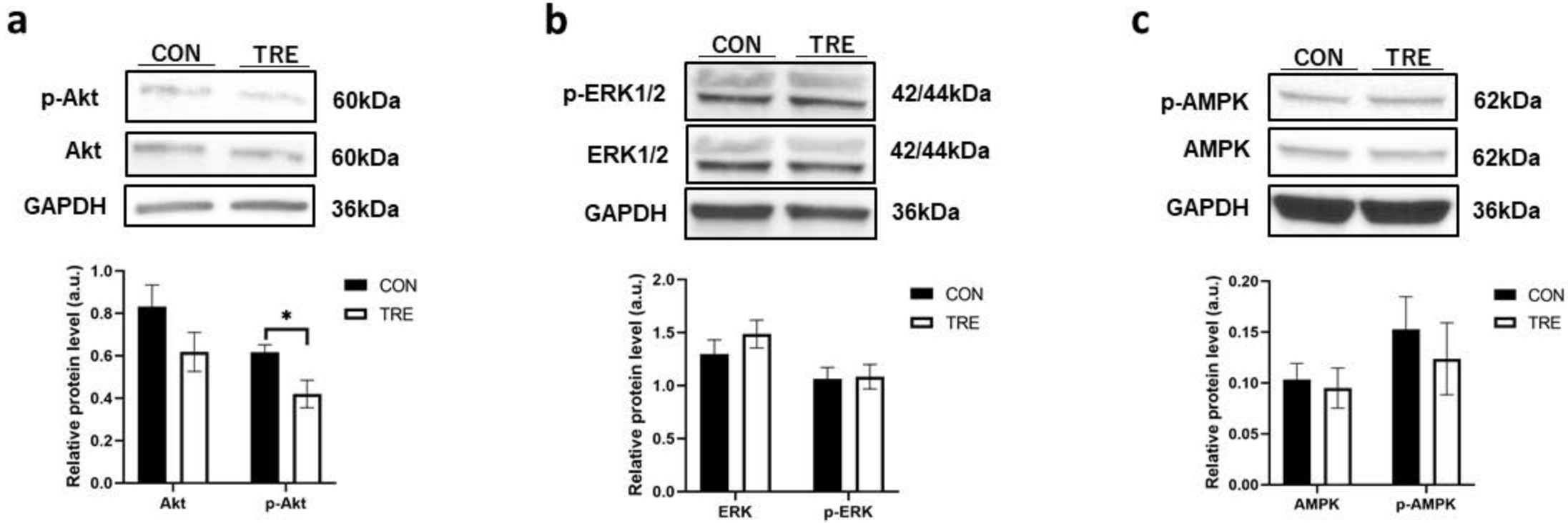

a
CON
TRE
p-eNOS
140kDa
GAPDH
36kDa
p-eNOS/GAPDH (a.u.)
0.6
0.4
0.2
0.0
CON
TRE
b
CON
TRE
Dimer
280kDa
Monomer
140kDa
eNOS
dimer / monomer (a.u.)
2.0
1.5
1.0
0.5
0.0
*
CON
TRE

Fig. 5


a
CON
TRE
p-Akt
60kDa
Akt
60kDa
GAPDH
36kDa
Relative protein level (a.u.)
1.0
0.8
0.6
0.4
0.2
0.0
*
CON
TRE
Akt
p-Akt
b
CON
TRE
p-ERK1/2
42/44kDa
ERK1/2
42/44kDa
GAPDH
36kDa
Relative protein level (a.u.)
2.0
1.5
1.0
0.5
0.0
CON
TRE
ERK
p-ERK
c
CON
TRE
p-AMPK
62kDa
AMPK
62kDa
GAPDH
36kDa
Relative protein level (a.u.)
0.20
0.15
0.10
0.05
0.00
CON
TRE
AMPK
p-AMPK

# Fig. 6

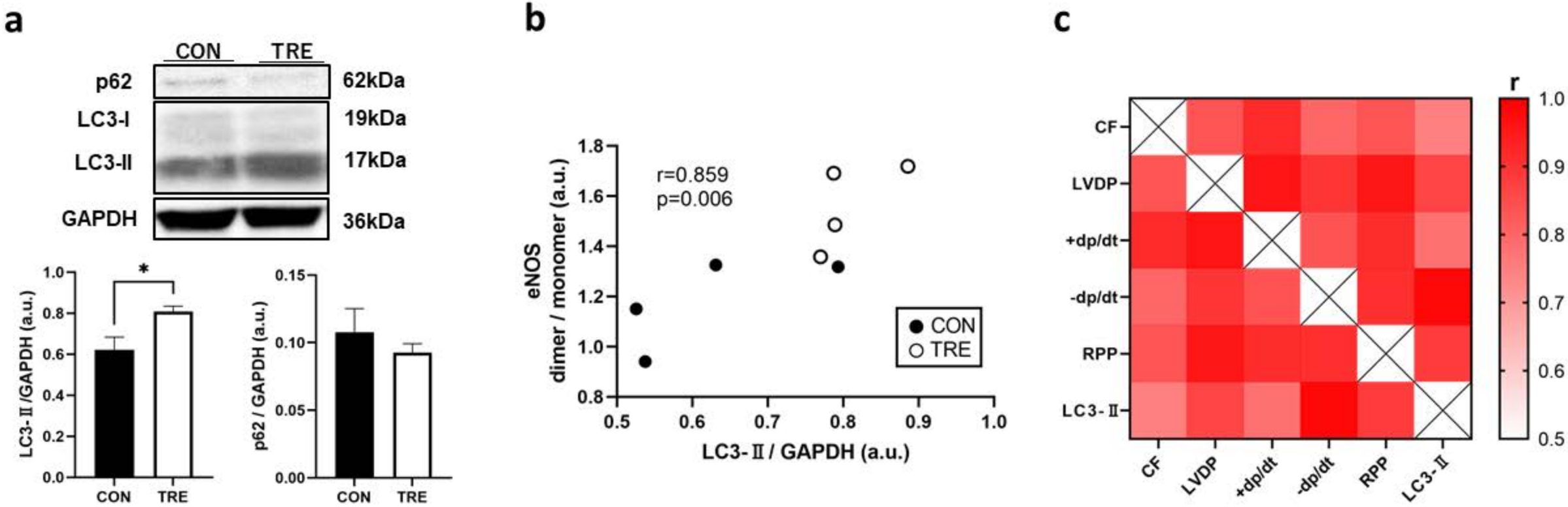

a
CON
TRE
p62
62kDa
LC3-I
19kDa
LC3-II
17kDa
GAPDH
36kDa
LC3-Ⅱ/GAPDH (a.u.)
*
CON
TRE
p62 / GAPDH (a.u.)
CON
TRE
b
r=0.859
p=0.006
eNOS
dimer / monomer (a.u.)
CON
TRE
LC3-Ⅱ / GAPDH (a.u.)
c
r
CF
LVDP
+dp/dt
-dp/dt
RPP
LC3-Ⅱ

Fig. 7

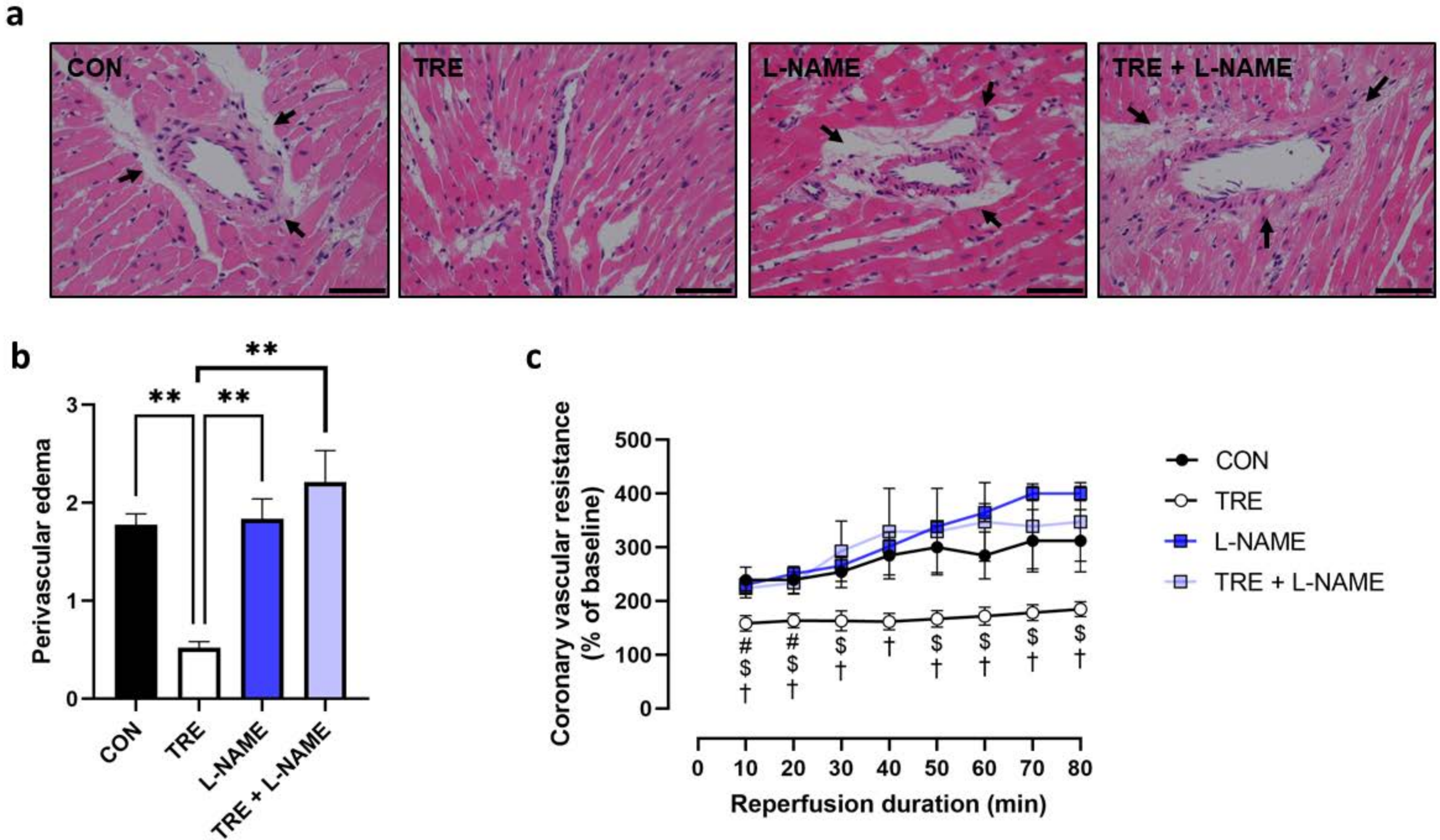
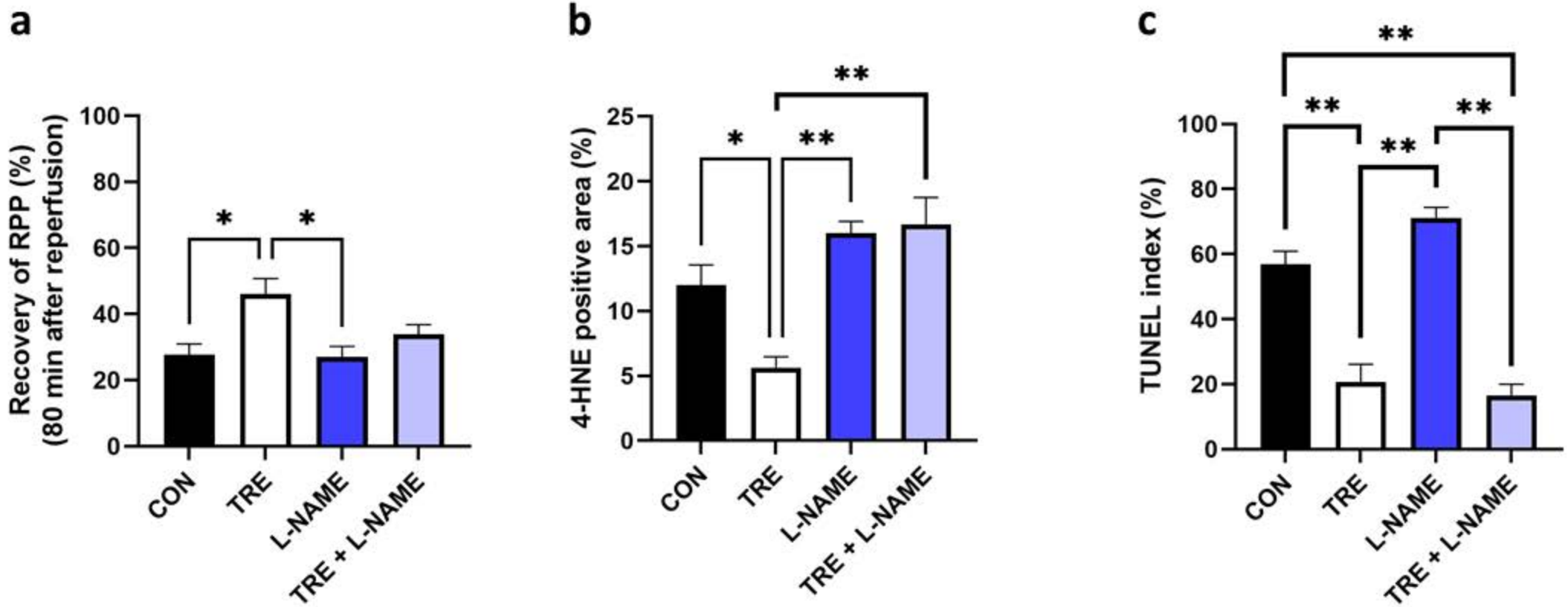

a
CON
TRE
L-NAME
TRE + L-NAME
b
Perivascular edema
0
1
2
3
**
**
**
CON
TRE
L-NAME
TRE + L-NAME
c
Coronary vascular resistance (% of baseline)
0
100
200
300
400
500
0
10
20
30
40
50
60
70
80
Reperfusion duration (min)
CON
TRE
L-NAME
TRE + L-NAME

Fig. 8


a
Recovery of RPP (%)
(80 min after reperfusion)
100
80
60
40
20
0
*
*
CON
TRE
L-NAME
TRE + L-NAME
b
4-HNE positive area (%)
25
20
15
10
5
0
*
**
**
CON
TRE
L-NAME
TRE + L-NAME
c
TUNEL index (%)
100
80
60
40
20
0
**
**
**
**
CON
TRE
L-NAME
TRE + L-NAME