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*Original article*

## **Trehalose Preconditioning for Transient Global Myocardial Ischemia in Rats**

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## **Abstract**

Autophagy is an intracellular pathway that degrades unnecessary proteins and organelles and provides energy substrates during cellular ischemic conditions. Although pharmacological myocardial preconditioning with an autophagy inducer has been reported to protect cells against ischemic reperfusion (I/R), the effects of preconditioning using naturally occurring substances are still unknown. We aimed to examine whether autophagic preconditioning with trehalose improves cardiac function after myocardial stunning by global ischemia in rats. Rat hearts were perfused by oxygenized Krebs Henseleit (KH) solution in Langendorff system. Ten rats were randomized into the following two groups according to the perfusates during the preconditioning: control (KH solution only, n=5) and trehalose (KH + 2% trehalose, n=5). After the 35-minute preconditioning period and subsequent 20 minutes of global ischemia, the hearts were reperfused for 60 minutes. Cardiac function was assessed during the reperfusion. To evaluate autophagy, myocardial protein expression of microtubule-associated protein light chain 3 (LC3) II was evaluated by western blotting. During I/R, a systolic functional parameter, maximum dP/dt was significantly higher; meanwhile, coronary flow tended to be higher in the trehalose group than in the control group. Myocardial LC3-II expression after preconditioning was higher in the trehalose group than in the control group and decreased to the control level after I/R. In conclusion, in a rat model of global myocardial ischemia, trehalose preconditioning improved cardiac function during I/R. Further studies would be needed to identify the mechanism and effects of trehalose preconditioning.

**Key words:** trehalose, preconditioning, autophagy, global myocardial ischemia

## **Introduction**

The outcomes of cardiac surgery have improved since 1970 because of the development of myocardial protection by cardioplegia. However, myocardial dysfunction after global myocardial ischemia is still a major problem after cardiac surgery. In heart transplantation, allograft ischemic time is linearly associated with an increased risk of death [1]. In general cardiac surgery, ischemic time affects mortality even in patients with preserved cardiac function [2]. Therefore, in the past few decades, several measures for improving myocardial protection during global ischemia have been investigated.

One of the greatest advances in myocardial protection from ischemia was the discovery of ischemic preconditioning by Murry et al. in 1986 [3]. They investigated myocardial infarction by left circumflex artery (LCX) occlusion in a dog model and reported that intermittent ischemia of LCX for 5 minutes drastically limited infarct size compared to control. However, intermittent ischemia cannot be routinely performed clinically due to the risk of coronary artery injury by mechanical occlusion. Therefore, non-mechanical preconditioning is the preferred technique if it has the same intracellular effects as mechanical ischemic preconditioning.

One of the important intracellular mechanisms of ischemic preconditioning has been reported to be “autophagy” [4]. The meaning of the word ‘autophagy’ is “self-eating”, and it is a fundamental intracellular biological pathway evolutionarily conserved from yeast to mammals. Autophagic digestion of unnecessary proteins and organelles provides amino and fatty acids as recycling substrates for adenosine triphosphate (ATP) production. During autophagic induction, a small vesicular sac called a phagophore elongates and subsequently encloses a portion of the cytoplasm. The phagophore is then transformed into an autophagosome, a double-membraned structure. The autophagosome fuses with a lysosome,

forming an autolysosome, in which enclosed materials are degraded. Intracellular energy, i.e., ATP, can be preserved by autophagy during cardiomyocyte ischemia [5]. Rapamycin, which induces autophagy by inhibiting mammalian target of rapamycin (mTOR), has protective effects against global ischemia. Preconditioning with rapamycin reduced infarct size by over 60% compared to controls in mice [6]. However, rapamycin is a type of immunosuppressant with side effects and would not be appropriate in the setting of cardiac surgery.

Trehalose, another autophagy inducer, is a naturally occurring, nontoxic disaccharide of glucose. Trehalose supplementation has been shown to activate autophagy and be effective for attenuating cardiac remodeling after myocardial infarction [7]. However, the role of trehalose in global myocardial ischemia is still unknown. We hypothesized that autophagic preconditioning by trehalose improves cardiac function after global myocardial ischemic reperfusion (I/R) in rats. The aim of this study was to clarify the relationship between trehalose preconditioning and cardiac function after transient I/R.

## **Methods**

### ***Experimental protocol***

Ten-week-old male Sprague-Dawley rats weighing 275–370 g were fed standard meals and city water. Rats were anesthetized by intramuscular ketamine (90 mg/kg) and xylazine (10 mg/kg) and euthanized by intraperitoneal injection of sodium pentobarbitone (150 mg/kg). After injection of heparin (100 IU) into the inferior vena cava, the hearts were rapidly excised and trimmed in ice-cold Krebs Henseleit (KH) solution. The ascending aorta was cannulated and secured by a suture. Within 3 minutes after euthanization, the hearts were retrogradely perfused by KH solution oxygenated by gassing with 95% O<sub>2</sub>/5% CO<sub>2</sub> at 37°C using a Langendorff system (LaboSupport, Osaka, Japan). The concentrations of substances in KH

solution were as follows (mmol/L): Na, 128; K, 5.0; Mg, 1.3; Ca, 2.5; glucose, 5. The constant perfusion pressure was set at 80 mmHg.

Rats were subjected to the experimental protocol shown in **Fig. 1**. After the 35-minute preconditioning period using the different solutions and a subsequent 20-minute period of global ischemia at room temperature, the hearts were reperfused for only 60 minutes with KH solution [6]. Ten rats were randomized into the following two groups during the preconditioning according to the perfusates: control (KH solution only, n=5) and trehalose (KH + 2% trehalose, n=5). Cardiac functional parameters were assessed immediately after the preconditioning period and every 5 minutes during the 60-minute reperfusion period. Finally, the left ventricles were cryopreserved to evaluate autophagic activation after I/R by western blotting. Moreover, to assess myocardial autophagic activation just after trehalose preconditioning, another 8 rats were euthanized (control, n=4; trehalose, n=4). (**Fig. 1**). Trehalose were purchased from HAYASHIBARA (Okayama, Japan).

### ***Cardiac functional assessment***

The following cardiac functional parameters were assessed as average values of 20 consecutive beats: heart rate; left ventricular developed pressure (LVDevP), defined as the difference between maximum LV pressure and end-diastolic pressure; maximum and minimum dP/dt; and rate pressure product, defined as LVDevP multiplied by heart rate. A fluid-filled 0.06-mL latex balloon (ADInstruments, Dunedin, NZ), connected to a pressure transducer, was inserted into the left ventricle and inflated to provide a minimum pressure between 5 and 15 mmHg. The pressure data were recorded using PowerLab (ADInstruments) and analyzed using LabChart (ADInstruments). Coronary flow was measured as the volume of drainage per minute from the coronary sinus. All procedures were conducted according to

the Hokkaido University Manual for Implementing Animal Experimentation and were 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).

### ***Immunoblotting***

Autophagic markers were microtubule-associated protein light chain 3 (LC3) II and p62/sequestosome-1 (SQSTM1). When autophagy is activated, LC3-I (cytosolic form) converts to LC3-II (membrane-bound lipidated form), which subsequently attaches to the autophagosomal membranes. Therefore, LC3-II reflects the number of autophagosomes and is commonly used as an autophagic marker. However, increase of LC3-II could overestimate the autophagic activation by possible suppression of its degradation pathway. Alternatively, p62/SQSTM1 is selectively incorporated into autophagosomes through direct binding to LC3 and is efficiently degraded by autophagy; the total cellular expression levels of p62/SQSTM1 decrease after autophagic activation [5]. Thus, increase of LC3-II accompanied by decrease of p62/SQSTM1 was considered to indicate autophagic activation. While there are numerous numbers of trigger pathways for autophagy, an intracellular energy sensor, adenosine monophosphate-activated protein kinase (AMPK) has been reported to activate autophagy by inhibiting mTOR [8]. The activation AMPK was measured as phospho-AMPK (p-AMPK) relative to total AMPK.

A semidry western blot apparatus (Mini-PROTEAN Tetra Cell, BIO-RAD, CA, USA) was used for detecting LC3-II and p62/SQSTM1. After sodium dodecyl sulfate-polyacrylamide gel electrophoresis (12% Mini-PROTEANTGX™, BIO-RAD, CA, USA), the proteins were blotted to polyvinylidene difluoride membranes and incubated with primary (anti-LC3B: abcam, Cambridge, UK; p62/SQSTM1, AMPK, and p-AMPK [Thr172]: Cell

Signaling, MA, USA) and secondary antibodies (anti-rabbit IgG, Cell Signaling). The bands were semiquantified by chemiluminescence using JustTLC (Sweday, Sodra Sandby, Sweden). Subsequently, the membranes were dyed with naphthol blue black solution to normalize the band intensity.

### *Statistical analysis*

All data were presented as mean  $\pm$  standard error of the mean. Cardiac functional parameters between the control and trehalose groups were compared using t-test. Intragroup changes of cardiac functional parameters were analyzed using repeated measures one-way analysis of variance (ANOVA) with post hoc Dunnett's test. The differences were considered significant at a value of  $P < 0.05$ . SPSS version 26 (SPSS Inc. Chicago, Ill) was used for statistical analysis.

## **Results**

### *Cardiac functional parameters*

**Fig. 2** shows the cardiac functional parameters before ischemia and every 5 minutes during reperfusion. Before ischemia (after preconditioning), there was no difference in coronary flow, heart rate, rate pressure product, LVDevP, and maximum and minimum dP/dt between the control and trehalose groups.

From 30 to 60 minutes after I/R, coronary flow was higher in the trehalose group than in the control group, although there was no statistical significance ( $p = 0.10-0.15$ ). Coronary flow increased early after reperfusion in both groups (**Fig. 2A**). No difference existed in heart rate between the two groups (**Fig. 2B**). Heart rate increased early after reperfusion only in the control group. The rate pressure product was also comparable between the groups (**Fig.**



**2C**). Although there was no significant difference in LVDevP between the groups, LVDevP increased early after reperfusion (5-35 minutes) only in the trehalose group (**Fig. 2D**). The values of maximum dP/dt were significantly higher in the trehalose group than those in the control group during reperfusion (**Fig. 2E**), while the values of minimum dP/dt were comparable between the two groups (**Fig. 2F**). The maximum dP/dt increased during reperfusion only in the trehalose group.

#### ***Protein expression of LC3-II, p62/SQSTM1, and AMPK***

**Fig. 3** shows the protein expressions of LC3-II (**Fig. 3A**) and p62/SQSTM1 (**Fig. 3B**) after preconditioning and I/R. After preconditioning, the protein expression of LC3-II was significantly higher in the trehalose group than in the control group (**Fig. 3A**). Although there were no significant difference in the expressions of p62/SQSTM1, the trends were opposite to those obtained for LC3-II (**Fig. 3B**). The results of the two autophagic markers suggest that autophagy was activated after preconditioning and returned to the control level after I/R in the trehalose group.

**Fig. 4** shows the expression of AMPK (**Fig. 4A**), p-AMPK (**Fig. 4B**), and the ratio of p-AMPK/AMPK (**Fig. 4C**). There was no difference in AMPK, p-AMPK, and p-AMPK/AMPK both after preconditioning and I/R between the two groups. Thus, AMPK was not associated with the trehalose effects.

#### **Discussion**

We demonstrated for the first time that cardiac function after I/R in the trehalose preconditioning group was better than that in the control in a model of transient global myocardial ischemia in rats. Autophagy was activated after trehalose preconditioning and

decreased to the control level after I/R.

### ***Role of autophagy in myocardial I/R***

Autophagy is an intracellular degradation system; cytoplasmic substances are delivered to and degraded in the lysosome. The role of autophagy consist not only of degradation of unnecessary materials, but also production of new energy substrates for cellular homeostasis and renovation [9]. However, autophagy may have a “double-edged sword” biological function in myocardial I/R: essential and protective roles during ischemia and detrimental role during reperfusion.

During the ischemic phase, autophagy serves as an energy-recovering process and is involved in cardiomyocyte survival. Energy substrates, such as amino-acids, free fatty acids, and glucose, can be recycled by autophagy and used for ATP generation [10]. Alternatively, during the reperfusion phase, excessive induction of autophagy after I/R may contribute to autophagic cardiomyocyte death. This was evidenced by the observation that autophagy inhibition reduced cardiomyocyte death after I/R [11,12]. However, in previous studies, authors inhibited autophagy not only in the reperfusion phase, but also in the ischemic phase. Thus, the specific role of autophagy in the reperfusion phase is still unknown.

In the present study, autophagic activation before ischemia was significantly higher in the trehalose group than in the control group. After I/R, autophagic activation in the trehalose group decreased to the control level. We speculated that autophagic preconditioning with trehalose played a protective role in cardiomyocytes during ischemia. Further study is necessary to determine whether this role was caused primarily by autophagic activation with trehalose.

### ***Trehalose as an autophagy inducer***

In numerous numbers of triggers for autophagy activation, AMPK-mTOR-dependent and independent pathways have been reported. mTOR is a key modulator of autophagy. AMPK suppresses mTOR activity by interfering with GTPase activity, leading to autophagy activation. [13] The precise mechanisms for autophagy activation by trehalose remain unclear [14].

In hepatocytes, autophagy induced by trehalose reduced intracellular lipid droplets in mice; trehalose inhibited solute carrier 2A, a family of glucose transporters, and induced autophagy in an AMPK-dependent manner [15]. In our present study, p-AMPK/AMPK both after preconditioning and I/R was comparable between the groups. Thus, AMPK may not be associated with the change in autophagy by trehalose in our experimental model.

In a mouse model, autophagy inhibition by hyperactivation of mTOR induced cardiac dysfunction; trehalose significantly improved cardiac function through autophagic activation by an mTOR-independent pathway [16]. In another mouse model of chronic myocardial infarction, trehalose reduced cardiac remodeling and preserved cardiac function by promoting autophagy [7]; indeed, the authors reported that trehalose activated the transcription factor EB, which is included in the upregulation of autophagic genes.

### ***Clinical implications***

Trehalose has multiple functions, including prevention of vasospasm, as well as anti-inflammatory and antioxidant effects [17]. Because these properties are related to cardiac surgery, particularly cardiopulmonary bypass, trehalose might have beneficial effects in this setting. In the present study, trehalose preconditioning improved cardiac function after

myocardial stunning, which may contribute to a decrease in catecholamine use, reduction of operative time, and postoperative recovery. To apply trehalose preconditioning to clinical practice in cardiac surgery, further studies are needed to examine the efficacy of this technique for longer ischemic time or I/R after cardioplegic arrest.

Trehalose is a naturally occurring nontoxic disaccharide of glucose. Intravenous injection, rather than oral administration, would be appropriate because less than 0.5% of ingested trehalose is absorbed into the blood stream. Although data about the safety margin of plasma trehalose concentration are scarce, the plasma concentration of 0.14% was reported to be safe in rats (United States Patent No: US 9,125,924 B2). Further investigations are needed to examine the optimal plasma concentration of trehalose.

### ***Limitations***

There are several limitations to be addressed. First, although trehalose had a positive impact on cardiac function after I/R, it is uncertain whether this was dependent on autophagy. These results might be attributed to other effects of trehalose, e.g. anti-inflammatory and anti-oxidant effects [17]. Further study using autophagy inhibitors is needed to assess the mechanism of trehalose-preconditioning effects. Second, our model was myocardial stunning; our results cannot be directly applied to infarction models. Myocardial stunning is classically defined as temporary mechanical dysfunction persisting after episodes of I/R with the absence of irreversible histological damage [18]. In our rat model, the cardiac dysfunction after I/R was not evident. Because myocardial caspase-3 (an apoptosis marker) was not activated in both groups (data not shown), indicating myocardial stunning rather than infarction. A previous report also indicated that 20 minutes of global ischemia induces myocardial stunning in rats [19].

## **Conclusions**

In a rat model of global myocardial ischemia, cardiac function after I/R was improved by trehalose preconditioning. Autophagy was activated by trehalose preconditioning and subsequently returned to control levels after I/R. Further study is needed to examine the mechanism of trehalose-preconditioning effects.

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**Conflict of interest**

None.

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**Summary of authors' contributions**

Data collection, data analysis, drafting article: Norihiro Ando

Data analysis/interpretations, drafting article: Yasushige Shingu

Data collection: Kenichiro Suno

Critical revision of article, approval of article; Satoru Wakasa

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## Figure legends

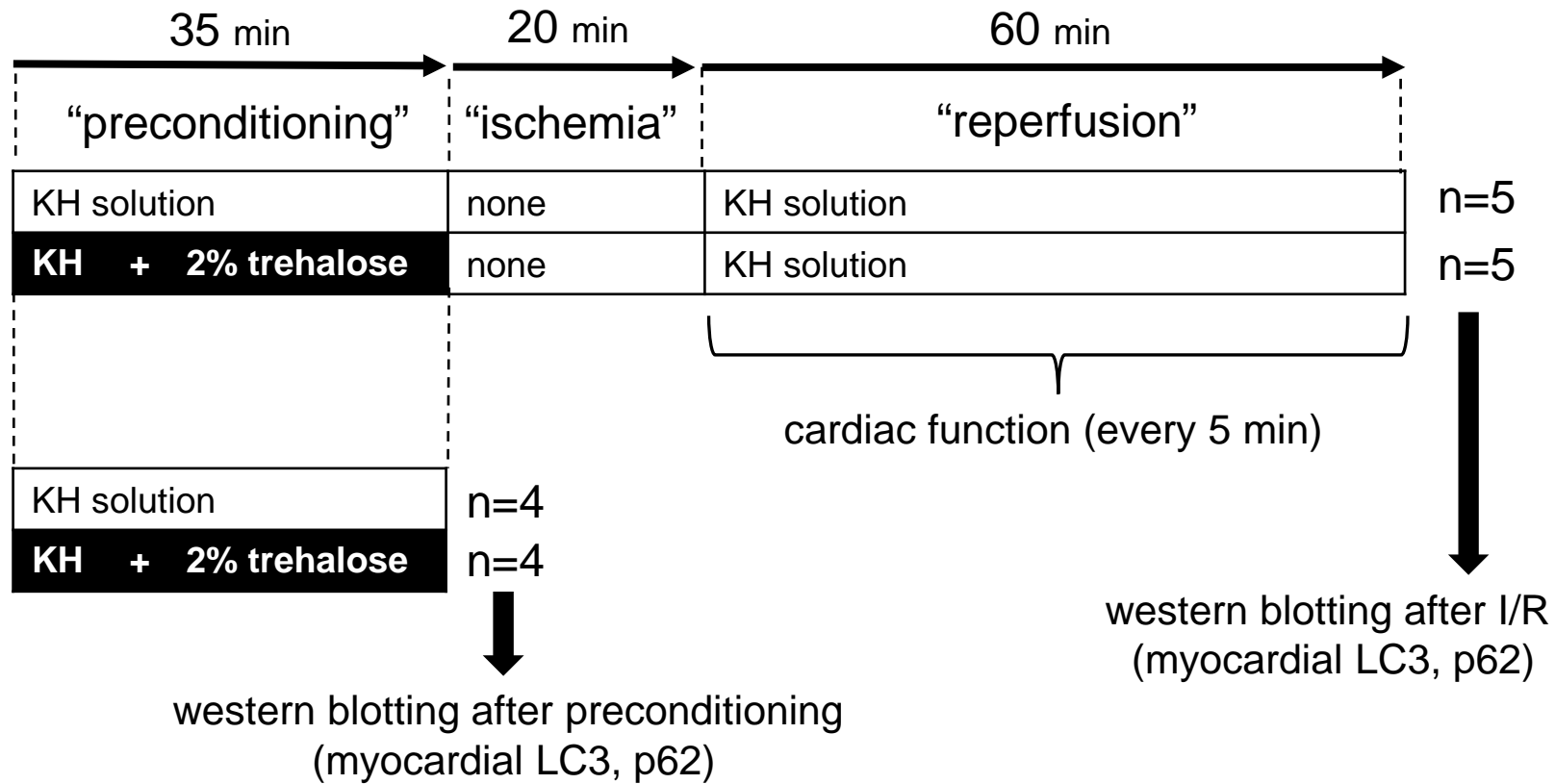
**Figure 1.** Experimental protocol. Rat hearts were perfused by KH solution (control) and KH + 2% trehalose (trehalose) oxygenated by gassing with 95% O<sub>2</sub>/5% CO<sub>2</sub> at 37°C using a Langendorff system. After 35-minutes preconditioning with the different solutions and subsequent 20-minutes global ischemia, the hearts were reperfused for 60 minutes with KH solution (n=10: control, n=5; trehalose, n=5). Cardiac functional parameters were recorded every 5 minutes. Other 8 rats (control, n=4; trehalose, n=4) were randomized to assess myocardial protein expressions by western blotting after 35-minutes preconditioning. KH, Krebs Henseleit; LC3-II, microtubule-associated protein light chain 3 II.

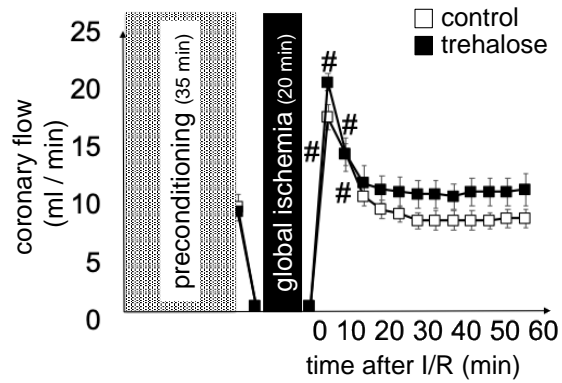
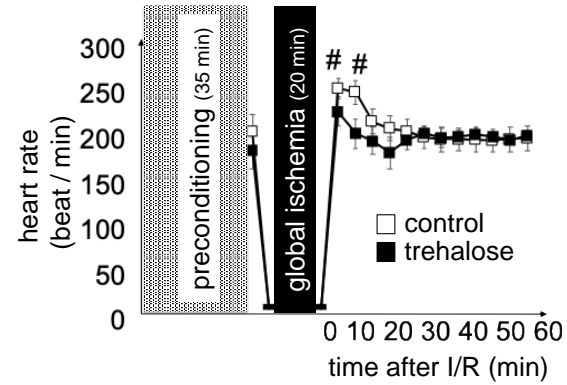
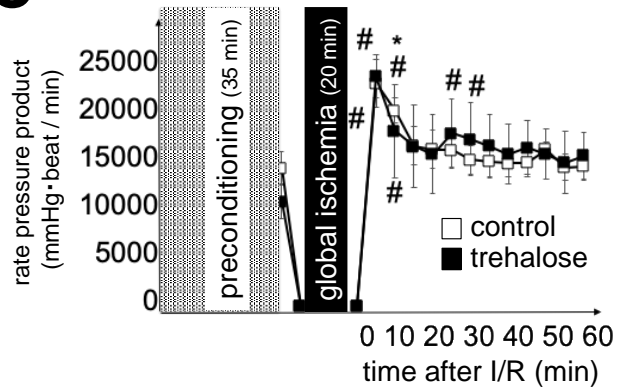
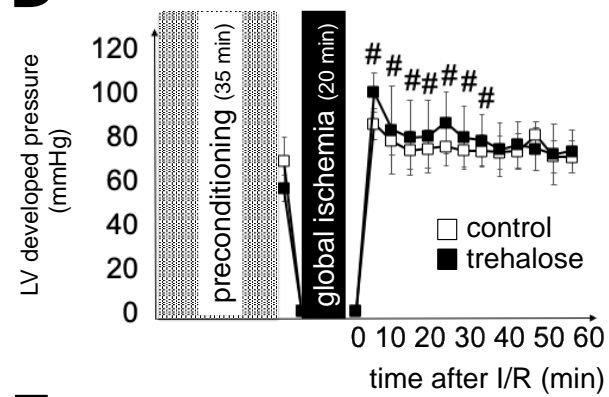
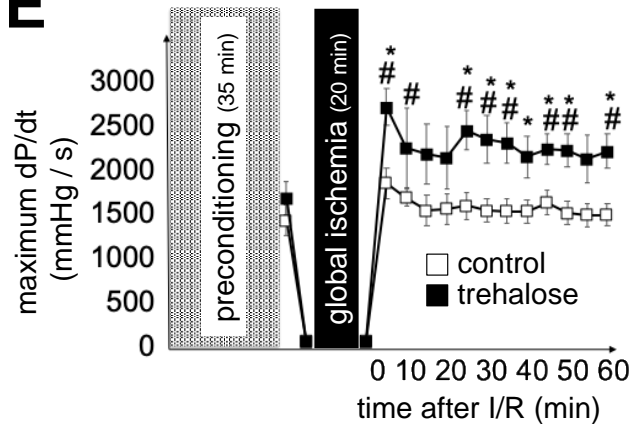
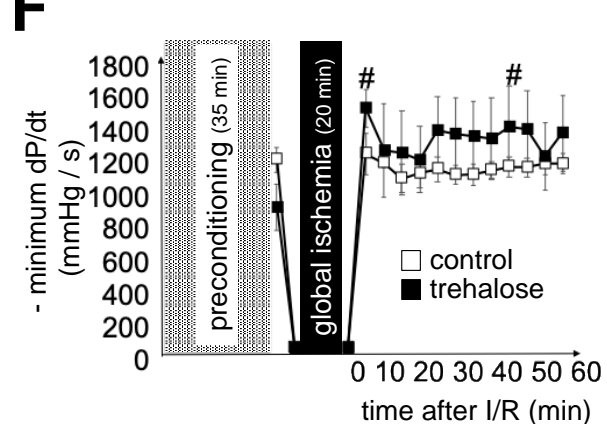
**Figure 2.** Changes of cardiac functional parameters every 5 minutes after 20-minutes global ischemia in control (white square) and trehalose group (black square): (A) coronary flow; (B) heart rate; (C) rate pressure product; (D) LV developed pressure; (E) maximum dP/dt; (F) minimum dP/dt. The squares indicate the means ± standard error of the means. The numbers of rats were 5 for each group. \*P<0.05 vs. control; #P<0.05 vs. after preconditioning. I/R, ischemic reperfusion; LV, left ventricular.

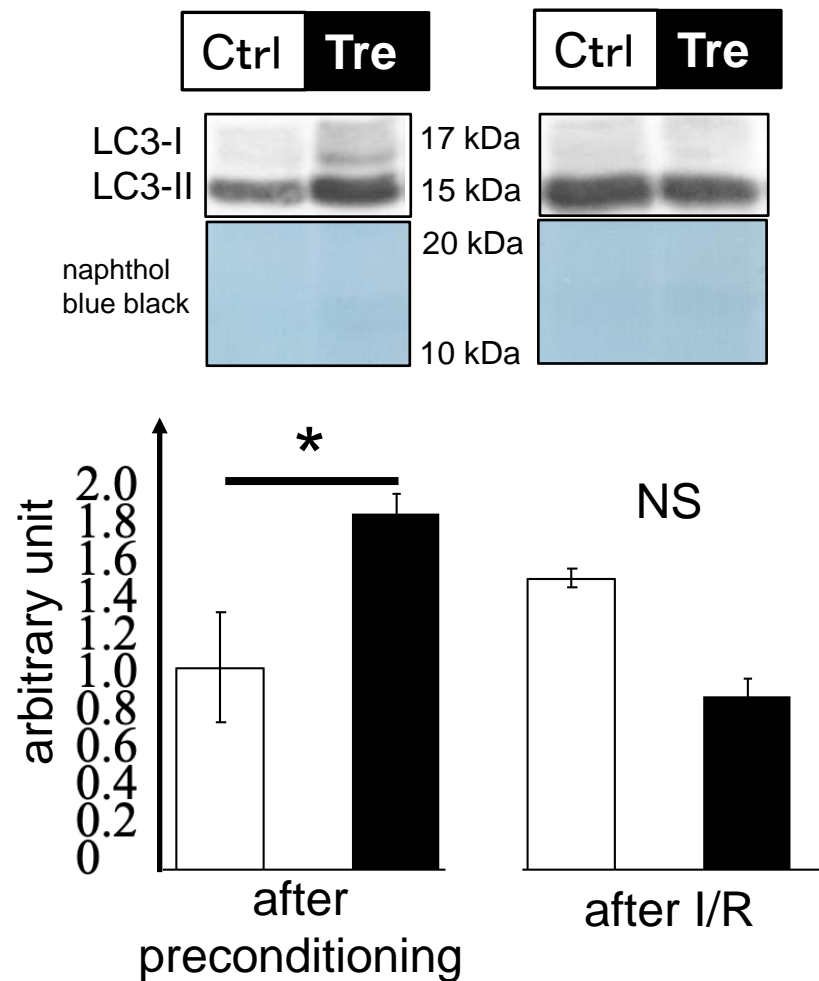
**Figure 3.** Protein expressions of autophagy markers after preconditioning and I/R: (A) LC3-II and (B) p62. The bar graphs indicate the means ± standard error of the means. The numbers of rats were 4 and 5 for each group after preconditioning and I/R, respectively. \*P<0.05 vs. control. I/R, ischemic reperfusion; LC3 II, microtubule-associated protein light chain 3 II; NS, not significant.

**Figure 4.** Protein expressions of AMPK (A), p-AMPK (B) and the ratio of p-AMPK/AMPK

(C). The bar graphs indicate the means  $\pm$  standard error of the means. The numbers of rats were 4 and 5 for each group after preconditioning and I/R, respectively. AMPK, adenosine monophosphate-activated protein kinase; p-AMPK, phospho-AMPK (Thr172); NS, not significant.



**A****B****C****D****E****F**

**A****B**