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ORIGINAL CONTRIBUTION

Remote ischemic preconditioning reduces myocardial ischemia–reperfusion injury through unacylated ghrelin-induced activation of the JAK/STAT pathway

Yasuaki Sawashita[1] · Naoyuki Hirata[1] · Yusuke Yoshikawa[1] · Hirofumi Terada[1] · Yasuyuki Tokinaga[1] · Michiaki Yamakage[1]

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Abstract

Remote ischemic preconditioning (RIPC) offers cardioprotection against myocardial ischemia–reperfusion injury. The humoral factors involved in RIPC that are released from parasympathetically innervated organs have not been identified. Previous studies showed that ghrelin, a hormone released from the stomach, is associated with cardioprotection. However, it is unknown whether or not ghrelin is involved in the mechanism of RIPC. This study aimed to determine whether ghrelin serves as one of the humoral factors in RIPC. RIPC group rats were subjected to three cycles of ischemia and reperfusion for 5 min in two limbs before left anterior descending (LAD) coronary artery ligation. Unacylated ghrelin (UAG) group rats were given 0.5 mcg/kg UAG intravenously 30 min before LAD ligation. Plasma levels of UAG in all groups were measured before and after RIPC procedures and UAG administration. Additionally, JAK2/STAT3 pathway inhibitor (AG490) was injected in RIPC and UAG groups to investigate abolishment of the cardioprotection of RIPC and UAG. Plasma levels of UAG, infarct size and phosphorylation of STAT3 were compared in all groups. Infarct size was significantly reduced in RIPC and UAG groups, compared to the other groups. Plasma levels of UAG in RIPC and UAG groups were significantly increased after RIPC and UAG administration, respectively. The cardioprotective effects of RIPC and UAG were accompanied by an increase in phosphorylation of STAT3 and abolished by AG490. This study indicated that RIPC reduces myocardial ischemia and reperfusion injury through UAG-induced activation of JAK/STAT pathway. UAG may be one of the humoral factors involved in the cardioprotective effects of RIPC.

Keywords Remote ischemic preconditioning · Myocardial ischemia reperfusion injury · Ghrelin · Preconditioning

Introduction

Globally, the number of age-related diseases has tended to increase annually along with longer human lifespans. Ischemic heart disease is globally recognized as a life-threatening disease that requires immediate therapeutic intervention. Early revascularization is the definitive treatment of acute coronary syndrome to limit infarct size (IS) and improve mortality. However, reperfusion of the ischemic myocardium itself can conversely worsen myocardial injury, which is known as myocardial ischemia–reperfusion injury [42]. Myocardial ischemia–reperfusion injury is a potentially preventable phenomenon that can be treated by both pharmacologic and non-pharmacologic interventions [32]. Murry et al. first demonstrated that brief episodes of non-lethal ischemia and reperfusion of the heart before sustained ischemia could limit myocardial IS in dogs. This cardioprotective effect was defined as ischemic preconditioning (IPC) [62]. Subsequently, local IPC was applied for elective surgical procedures due to its clinical benefit [33]. This kind of cardioprotection can be induced by ischemic conditioning locally in the heart, as well as in tissues and organs remote from the heart. Remote ischemic conditioning can be induced before (pre-) or during (per-) ongoing

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✉ Yasuaki Sawashita
y.sawashita@sapmed.ac.jp

[1] Department of Anesthesiology, Sapporo Medical University School of Medicine, S-1, W-16, Chuo-ku, Sapporo, Hokkaido 060-8543, Japan

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myocardial ischemia or after reperfusion (postconditioning), all of which have been shown to protect against myocardial ischemia–reperfusion injury in many experimental studies [12, 45, 46, 69]. However, the translation from animal experiments to clinical situations for patients with acute myocardial infarction has not been successful. In clinical trials of undergoing coronary artery bypass grafting, the cardioprotective effects of remote ischemic conditioning have been contentious [28, 51, 60]. In some studies, remote ischemic conditioning was completely unable to improve clinical outcomes in patients receiving primary percutaneous coronary intervention (PPCI) against myocardial infarction [18, 30, 88]. These results that are contrary to those of experimental studies might be attributed to a variety of factors [16, 43, 47, 89]. Therefore, it is quite possible that elucidation of the unexplained mechanism of remote ischemic conditioning will lead to clinical benefits.

The cardioprotective effect of remote ischemic conditioning reportedly involves humoral factors, neuronal signaling systems and their interaction [27, 48, 67]. Across species, humoral factors have been shown to be involved in activation of signal transduction [78]. As the humoral factors were characterized as having a size of < 12–14 kDa, and being hydrophobic, lyophilized and thermolabile, they were presumed to be a peptide or protein [38, 53, 74, 76]. The cardioprotective effect of remote ischemic conditioning is also based on the neuronal system, including the spinal cord, sympathetic and parasympathetic nervous systems [52, 59, 86]. Several animal studies reported the important role of intactness of vagal nerve in the cardioprotective effect of remote ischemic conditioning [21, 61]. This phenomenon has been demonstrated by a previous study that showed that nitric oxide (NO) released by the stimulus of RIPC modulates the secretion of acetylcholine from parasympathetic nerve via presynaptic activity [31]. In particular, the cardioprotective effect of remote ischemic conditioning was eliminated by sectioning of bilateral cervical vagal nerves and the posterior gastric branch of the vagal nerve [59, 86]. Additionally, interactions between the humoral factors and neuronal systems have been reported such as the relationship between activation of the vagal nerve and glucagon-like peptide-1 (GLP) released from the gut as well as between vagal nerve and spleen [4, 5, 55, 65]. These results indicate that the mechanism may involve unknown humoral factors, vagal nerve activity and the visceral organs.

Ghrelin is a growth hormone-releasing peptide released by the stomach that exists in the circulation in two forms: acylated ghrelin (AG) and unacylated ghrelin (UAG) [39, 49]. AG affects food intake and growth hormone secretion, whereas UAG does not. Total ghrelin concentration in circulation is the sum of AG and UAG. Previous studies found that both AG and UAG have cardioprotective effects via the activation of certain cell signaling pathways [2, 40, 50, 81]. Some studies demonstrated a correlation between growth hormone family and remote ischemic conditioning via janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathway [15, 48, 66]. However, it has never been proved whether ghrelin, which is released by the stomach in response to vagal activity, exerts its cardioprotective effects via activation of STAT in remote ischemic conditioning. We hypothesized that ghrelin might be one of the humoral factors involved in remote ischemic conditioning.

The aim of the present study was to elucidate whether or not ghrelin is one of the humoral factors contributing to the cardioprotective effects of remote ischemic preconditioning (RIPC). We focused on the secretion of ghrelin following the peripheral stimulus of RIPC procedures via acceleration of vagal activity. Furthermore, we investigated the association between the secreted ghrelin and activation of cell signaling pathways in cardiomyocytes.

Methods

Approval and preparation of animal experiments

All animal experiments and protocols were performed after the approval of the Institutional Animal Care and Use Committee of Sapporo Medical University (No. 17-117,118), and strictly adhered to the Guide for the Care and Use of Laboratory Animals (Institute for Laboratory Animal Research, 8th edition). Experiments were conducted in male Wistar rats aged 2–3 months. The rats were housed in our institutional animal facility in a temperature-controlled room (22–24 °C) under a 12-h light/12-h dark cycle with unlimited access to food and water.

Experimental protocol in vivo

Male Wistar rats were randomly assigned to the control (CON), RIPC, UAG, RIPC + AG490 (JAK2/STAT3 pathway inhibitor) and UAG + AG490 groups (Fig. 1). Myocardial ischemia–reperfusion injury was induced in all the rats in this in vivo model according to a previously described method, while they were positioned on a heating pad to maintain their body temperature at 37 °C [6, 77, 83]. Briefly, all the rats were anesthetized intraperitoneally with a mixture of three anesthetic agents, midazolam (2 mg/kg), butorphanol (2.5 mg/kg) and medetomidine (0.15 mg/kg), in accordance with previous reports [64]. Then, they were intubated with a 16-gauge cannula and were mechanically ventilated using a volume-controlled mode with the tidal volume set to 1 mL/100 g at a breath rate of 60 breaths/min (Model 683 Small Animal Ventilator, Harvard Apparatus, Holliston, MA, USA). The tail vein was cannulated for drug infusion. Blood pressure was continuously monitored

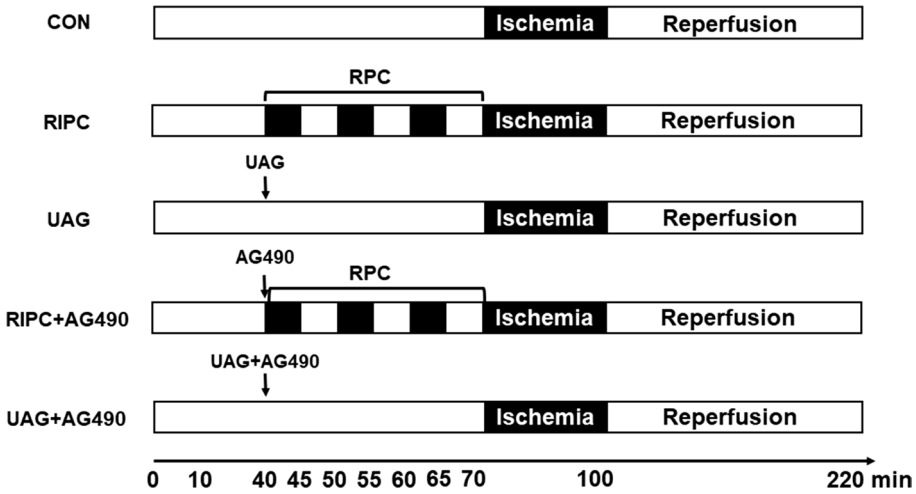

Fig. 1 Experimental protocol. All rats were subjected to 30 min of left anterior descending (LAD) coronary artery ligation followed by 120 min of reperfusion. The control group (CON) only underwent myocardial ischemia–reperfusion. The remote ischemia preconditioning (RIPC) group received RIPC procedures (RPC) on two of their four limbs, which involved three cycles of 5 min ischemia and 5 min reperfusion. Rats in the unacylated ghrelin (UAG) group received 0.5 mcg/kg of UAG administered intravenously 30 min before LAD coronary artery ligation. Rats in the RIPC + AG490 and UAG + AG490 groups received 3 mg/kg of AG490 intravenously in addition to the UAG injection or RIPC 30 min before LAD coronary artery ligation. At the end of the experimental protocol, hearts were collected and infarct size was assessed using 1% 2,3,5-triphenyltetrazolium chloride staining. CON, control group; RIPC, remote ischemic preconditioning; RPC, remote ischemic preconditioning procedures; UAG, unacylated ghrelin; AG490, JAK2/STAT3 pathway inhibitor

through a cannula inserted into the left femoral artery. For assessment of echocardiograms (ECG) (lead II), needles were inserted subcutaneously into the anterior thorax. Myocardial ischemia–reperfusion injury was established as follows. The left anterior descending (LAD) coronary artery was ligated using 6–0 polypropylene. Then, the heart was subjected to 30 min of ischemia followed by 120 min of reperfusion. Successful myocardial infarction was confirmed by observation of pallor of the heart and apparent ST segment elevation on the ECG recorded by the PowerLab system (ADInstruments, Sydney, Australia). Rats in the CON group only underwent myocardial ischemia–reperfusion injury. In the RIPC group, RIPC procedures were performed on the right forelimb and hindlimb, with three cycles of ischemia for 5 min and reperfusion for 5 min, just before ligation of the LAD coronary artery. For RIPC, the right forelimb and hindlimb were encircled with neonatal blood pressure cuffs. The cuffs were inflated to 200 mmHg to achieve pallor of the limbs. In the UAG group, UAG (0.5 mcg/kg) (031-33, Phoenix Pharmaceuticals, Belmont, CA, USA) was administered intravenously 30 min before the beginning of LAD coronary artery ligation. In the RIPC + AG490 and UAG + AG490 groups, in addition to the RIPC or UAG administration, respectively, AG490 (3 mg/kg) (Abcam, Cambridge, MA, USA) was also intravenously infused 30 min before LAD ligation [22, 44]. Blood sampling was performed for the measurement of plasma concentrations of AG and UAG both pre and post the RIPC procedure, and before and 30 min after administration of UAG and/or AG490. The volume of blood collected at each sampling was 0.5 mL, samples being obtained from the cannulated left femoral artery. The same volume of normal saline was administered into the tail vein after each blood sampling. The blood samples were mixed with aprotinin and ethylenediaminetetraacetic acid to measure plasma levels of ghrelin. All the collected blood samples were centrifuged at 1500*g* at 4 °C for 15 min to extract the plasma, which was subsequently stored at − 80 °C for enzyme-linked immunosorbent assay (ELISA).

Infarct size determination

IS was assessed using a 1% 2,3,5-triphenyltetrazolium chloride (TTC) dying manner described in a previous study [7]. Briefly, at the end of the experimental protocols, LAD coronary artery re-occlusion was performed, and 4% Evans Blue was injected through the tail vein to determine the normal coronary flow region of the left ventricle (LV), in order to calculate the anatomic area at risk (AAR). Next, the heart was excised without pain for euthanasia, and the right atrium, right ventricular free wall and the adherent tissue were removed. The LV was cut into 2-mm-thick slices and the slices were incubated in a 1% solution of TTC in phosphate-buffered saline (pH 7.4) for 15 min at 37 °C, fixed in 10% formalin for 20 min and photographed. The AAR and IS were evaluated in a blinded manner using a planimetry method and ImageJ software (National Institutes of Health, Bethesda, MD, USA). The AAR and IS were expressed as percentages relative to LV area and AAR, respectively.

Measurements of AG and UAG concentrations

Plasma concentrations of both AG and UAG were determined using the ELISA method and a commercially available kit (LSI Medience, Tokyo, Japan). Data evaluations were performed spectrophotometrically using a standard 96-well plate reader at a 450 nm wavelength (Sunrise™ reader, Tecan Group Ltd., Männedorf, Switzerland).

Western blotting

We examined the modulation of phosphorylation of $STAT3_{tyr705}$, which may play a crucial role in the cardioprotective effects of RIPC and UAG, as in previous studies in pigs [36, 55, 78]. For this, left ventricular apex tissue was sampled at two time points: just prior to ischemia and 10 min after reperfusion from separate rats. The rats were euthanized after collection of the myocardial tissue. Total protein was extracted with ice-cold buffer, and the concentrations of protein were detected with a BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA) [41]. Western blotting was performed as described previously [90]. Briefly, equal amounts of proteins were separated by 7.5–12.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA, USA). After blocking, the membranes were incubated with antibodies directed against STAT3 (#12640, 1:1000; Cell signaling Technology, Beverly, MA, USA) and the phosphorylated forms of $STAT3_{tyr705}$ (#9138, 1:1000; Cell signaling Technology, Beverly, MA, USA). All blots were analyzed in a blinded manner.

Statistical analysis

Based on a previous study, a sample size of six rats in each group was deemed necessary to detect a 25% reduction in IS, which was considered to be appropriate ($\alpha = 0.05$, $1 - \beta = 0.8$, two-tailed) and clinically effective by the G*Power 3.1 statistical power analysis program (Heinrich-Heine-University, Düsseldorf, Germany) [8]. All data were tested for normal distribution using the Shapiro–Wilk test. Data are presented as mean ± standard error of the mean (SEM). AAR, IS and ratio of total STAT3 and phosphorylation of $STAT3_{tyr705}$ among all groups were analyzed using one-way analysis of variance (ANOVA) followed by the Tukey post hoc test. The levels of AG and UAG in the plasma at different time points and hemodynamic status were analyzed by two-way ANOVA for repeated measures (protocol, time). All statistical analyses of data were performed using GraphPad Prism 7.0 software (GraphPad Software, La Jolla, CA, USA). Statistical differences were considered significant at a value of $P < 0.05$.

Results

Hemodynamic status

The rats' hemodynamic status is shown in Table 1. There were no significant differences in HR between the five

Table 1 Hemodynamic status of the rats

	Baseline	Ischemia	Reperfusion	
		15 min	30 min	120 min
HR (bpm)				
CON	258.6 ± 8.5	290.0 ± 8.9	272.9 ± 12.7	316.0 ± 15.0
RIPC	286.6 ± 26.6	246.6 ± 9.6	230.1 ± 13.3	245.3 ± 28.4
UAG	266.6 ± 18.6	272.8 ± 20.9	276.0 ± 10.9	317.0 ± 15.3
RIPC + AG490	296.6 ± 9.4	281.3 ± 15.9	291.0 ± 14.6	305.4 ± 16.9
UAG + AG490	265.0 ± 19.9	279.6 ± 23.8	289.1 ± 17.5	310.9 ± 29.2
MBP (mmHg)				
CON	137.9 ± 3.9	96.9 ± 7.0*	66.9 ± 4.3*	64.9 ± 2.6*
RIPC	117.2 ± 4.4	76.9 ± 10.7*	58.0 ± 7.1*	58.4 ± 7.0*
UAG	148.1 ± 5.3	94.6 ± 7.1*	64.9 ± 2.8*	69.9 ± 4.1*
RIPC + AG490	123.0 ± 6.9	83.3 ± 11.3*	65.7 ± 2.9*	63.4 ± 3.3*
UAG + AG490	123.1 ± 5.5	83.7 ± 6.2*	66.0 ± 2.3*	69.9 ± 2.1*

Data are shown as mean ± standard error of the mean (SEM)

HR, heart rate; CON, control group; RIPC, remote ischemic preconditioning group; UAG, unacylated ghrelin; AG490, JAK2/STAT3 pathway inhibitor; MBP, mean blood pressure

*$P < 0.05$ vs. baseline in each group, $n = 8$–10, as assessed using two-way ANOVA followed by Tukey post hoc test

groups at baseline, and during ischemia and reperfusion. However, MBP decreased compared to baseline during ischemia and reperfusion in all groups ($P < 0.05$).

Area at risk and Infarct size

The area at risk was not significantly different between all groups (CON: 57.4% ± 2.4%, RIPC: 55.8% ± 2.3%, UAG: 54.7% ± 1.9%, RIPC + AG490: 57.9% ± 1.5%, UAG + AG490: 60.5% ± 2.1%, Fig. 2). IS was significantly reduced in the RIPC and UAG groups, as compared to that in the CON group (CON: 54.1% ± 4.2%, RIPC: 34.2% ± 3.6%, UAG: 33.8% ± 2.7%, $P < 0.05$ Fig. 3), while it remained similar to control levels in the RIPC + AG490 and UAG + AG490 groups (RIPC + AG490: 54.6% ± 5.0%, UAG + AG490: 55.0% ± 3.5%).

Plasma levels of acylated and unacylated ghrelin

Baseline levels of AG and UAG were comparable among groups. In all except the CON group, plasma levels of UAG were significantly increased by RIPC or UAG administration compared to the respective baseline levels (CON pre: 1,370.6 ± 53.5 pg/mL, CON post: 2,003.9 ± 83.8 pg/mL, RIPC pre: 1,626.6 ± 86.3 pg/mL, RIPC post: 2,681.6 ± 355.1 pg/mL, UAG pre: 1,653.7 ± 132.9 pg/mL, UAG post: 2,864.0 ± 288.9 pg/mL, RIPC + AG490 pre: 1,504.5 ± 191.1 pg/mL, RIPC + AG490 post: 2,460.2 ± 279.2 pg/mL, UAG + AG490 pre: 1,354.9 ± 173.1 pg/mL, UAG + AG490 post: 2,550.6 ± 117.0 pg/mL, $P < 0.05$, Fig. 4). There were, however, no significant differences in plasma levels of AG among the groups (Online resource. 1).

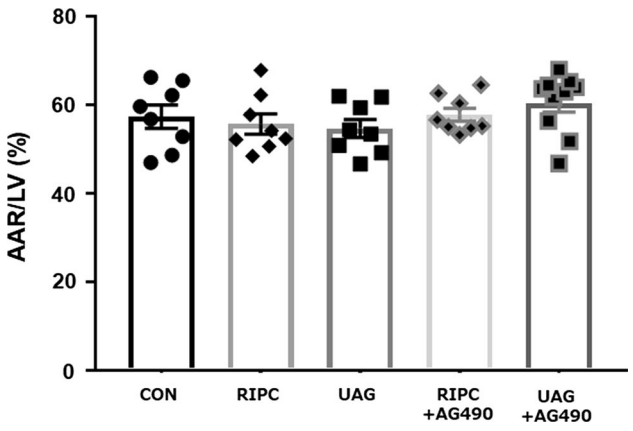

Fig. 2 Area at risk of infarction in the five groups. The area at risk is presented as a percentage relative to the area of the left ventricle. Data are shown as mean % ± SEM, $n = 8$–10 rats per group. Analysis by one-way ANOVA followed by Tukey post hoc test revealed no significant differences between groups. CON, control group; RIPC, Remote ischemic preconditioning group; UAG, unacylated ghrelin; AG490, JAK2/STAT3 pathway inhibitor

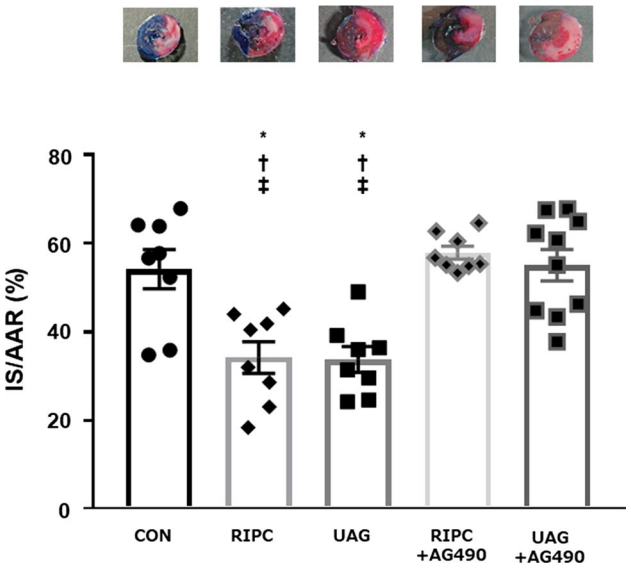

Fig. 3 Infarct size in the five groups. Infarct size is presented as a percentage relative to the area at risk. Data are shown as mean % ± SEM, $n = 8$–10 rats per group. $^{*}P < 0.05$ vs. CON, $^{\dagger}P < 0.05$ vs. RIPC + AG490, $^{\ddagger}P < 0.05$ vs. UAG + AG490, by one-way ANOVA followed by Tukey post hoc test. CON, control group; RIPC, Remote ischemic preconditioning group; UAG, unacylated ghrelin; AG490, JAK2/STAT3 pathway inhibitor

Western blotting

$STAT3_{tyr705}$ phosphorylation 10 min after reperfusion was higher than that after the experimental procedures in RIPC and UAG groups, but not in the other groups ($P < 0.05$, Fig. 5 and Online resource 2). Thus, phosphorylation of $STAT3_{tyr705}$ protein 10 min after reperfusion was abolished by administration of AG490. Additionally, phosphorylation of $STAT3_{tyr705}$ protein 10 min after reperfusion in RIPC group was significantly higher than that in UAG group.

Discussion

We showed that RIPC procedures increase plasma UAG levels and protect against myocardial ischemia–reperfusion injury via phosphorylation of $STAT3_{tyr705}$. Furthermore, we confirmed that the increase in plasma UAG levels resulting from administration of 0.5 mcg/kg UAG had similar cardioprotective effects as RIPC. It has previously never been demonstrated whether ghrelin exerts its RIPC-induced cardioprotective effects via activation of the STAT pathway. The present study indicated that UAG might be a crucial humoral factor in the cardioprotective effects of RIPC.

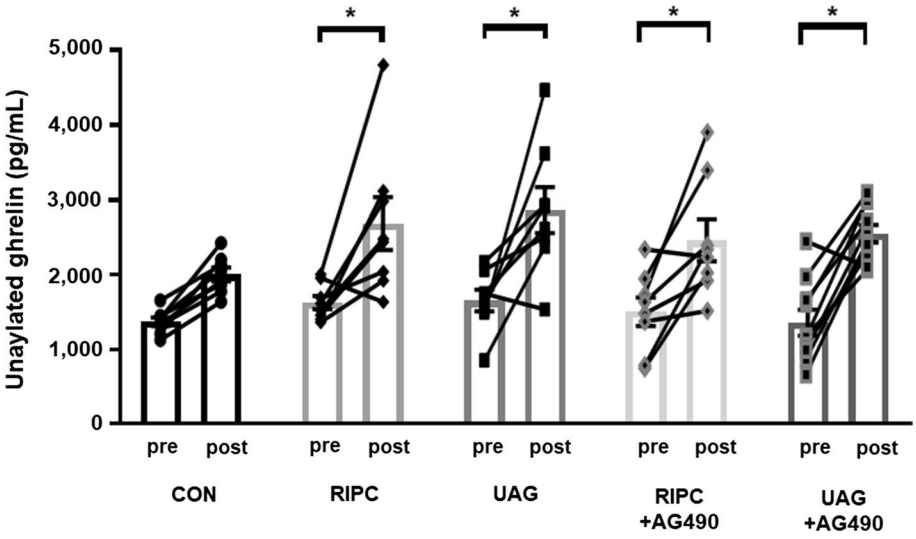

Fig. 4 Plasma concentrations of UAG in the five groups. Blood samples in each group were collected both before and after the RIPC procedure, and before and 30 min after administration of UAG and/or AG490. Data are shown as mean (pg/mL) ± SEM, $n = 8$–10 rats per group. *$P < 0.05$ vs. the 'pre' period in each group, as assessed by two-way ANOVA followed by Tukey post hoc test. CON, control group; RIPC, Remote ischemic preconditioning group; UAG, unacylated ghrelin; AG490, JAK2/STAT3 pathway inhibitor; pre, before RIPC or drug administration; post, 30 min after RIPC or drug administration

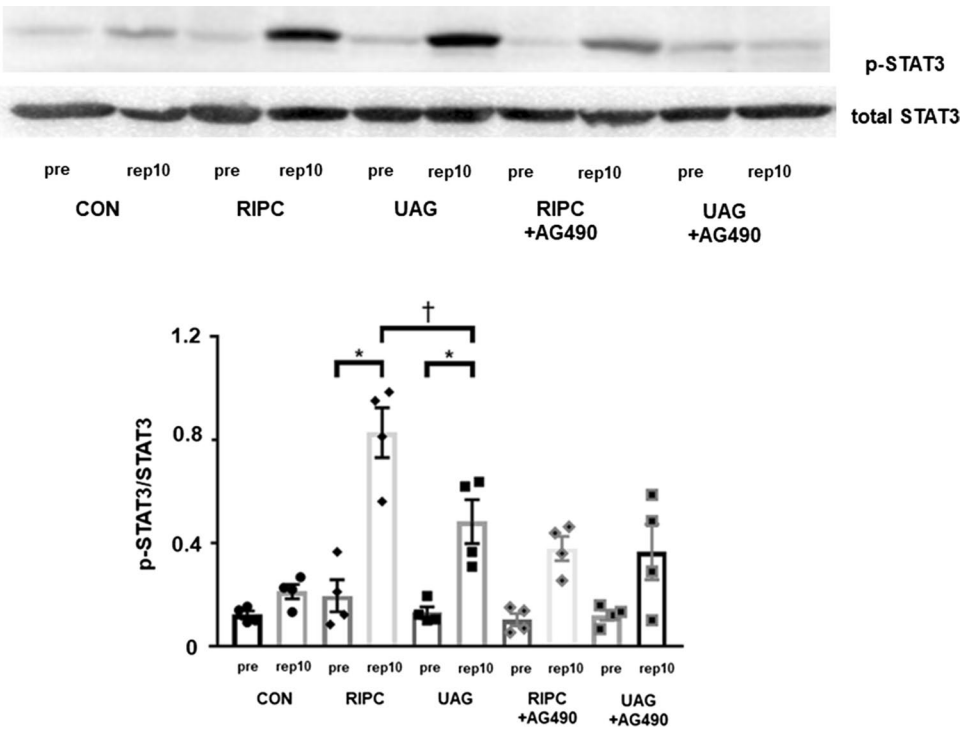

Fig. 5 Ratio of phosphorylated STAT3 and total STAT3 in rat myocardium sampled from the left ventricular apex pre-ischemia and after 10 min of reperfusion among the five groups. Data are shown as mean ± SEM, $n = 4$ per group. *$P < 0.05$ vs. 'pre' in each group, assessed by one-way analysis of variance (ANOVA) followed by Tukey post hoc test. †$P = 0.0112$ assessed by one-way ANOVA followed by Tukey post hoc test. CON, control group; RIPC, Remote ischemic preconditioning group; UAG, unacylated ghrelin; AG490, JAK2/STAT3 pathway inhibitor; pre, before RIPC or drug administration; rep10, 10 min after reperfusion in the left anterior descending coronary artery

Many researchers have tried to reveal the complex mechanisms involved in remote ischemic conditioning. They found that the mechanism is very complex and involves humoral factors, neuronal systems and their interactions [9, 59, 63, 67, 91]. Various experimental studies have shown different results and outcomes depending on species differences [3, 55, 61]. Therefore, the factors based on the mechanisms of remote ischemic conditioning are both common and controversial between animal species. Some studies in rodents and humans found that endothelium-derived NO produced peripherally by the stimulus of RIPC, which exists into circulation as nitrite, mediates the cardioprotective effect by

NO transfer into the cardiomyocytes [1, 14, 70, 75]. Indeed, NO serves RIPC cardioprotection as one of the humoral factors in rodents and humans, but not in pigs and rabbits [10, 68]. The neuronal systems of remote ischemic conditioning are mainly composed of sympathetic and parasympathetic nerves and the spinal cord [4, 27, 52, 57, 59, 86]. In particular, several experimental studies reported the important role of an intact vagal nerve underling the cardioprotective effect of RIPC [3, 21, 61, 71, 86]. These studies were supported by the evidence that NO promotes acetylcholine release from parasympathetic nerve fibers via presynaptic activation [31]. Although interactions between the humoral factors and neuronal systems have been reported such as the relationship between the vagal nerve and GLP-1 in rats, some studies indicated that there are no GLP-1 receptors on cardiomyocytes and that GLP-1 does not activate the cardiac vagal nerves [4, 5, 65, 74, 76]. On the other hand, Lieder HR et al. demonstrated that the cardioprotection of RIPC is based on the interaction between the vagal nerve and spleen in pigs and rats [55]. Interestingly, they found that vagotomy with RIPC in pigs did not eliminate the reduction in IS at all, and that the spleen critically releases humoral factors through vagal nerve-induced acetylcholine release. Some studies showed that the cardioprotective effect of RIPC was eliminated by sectioning of the vagal nerve in the neck bilaterally and of the posterior gastric branch of the vagal nerve in rabbits and rats [13, 59, 67]. A previous study additionally found that electrical stimulation of the posterior gastric branch of the vagal nerve imitated the cardioprotective effect of RIPC in rats [59]. This study suggested that the visceral organs innervated by the posterior gastric branch of the vagal nerve can produce and release humoral factors. However, the vagal nerve-linked humoral factors released by the stomach that contribute to RIPC-induced cardioprotection have not been determined. Our study focused on ghrelin as the potential humoral factor, since ghrelin is derived from gastric parietal cells via NO vagal nerve-induced acetylcholine release and might have cardioprotective effects against myocardial ischemia–reperfusion injury [59, 84]. The present study showed that the plasma concentrations of UAG, but not AG, were significantly elevated with RIPC procedures. In fact, previous studies have reported the association between ghrelin and vagal afferents [85, 87]. Veedfald et al. found that the total ghrelin concentration in vagotomized patients was decreased compared to that in patients with intact vagal innervation and after indirect vagal stimulation with food intake [85]. Williams et al. showed that the total ghrelin concentration in vagotomized or muscarine receptor blocker-administered rats was not increased compared to that in rats with intact vagal innervation under 48 h of food deprivation [87]. Thus, the secretion of ghrelin was suppressed by vagotomy or systemic administration of muscarinic receptor blockers. These studies might support the present hypothesis that NO released by the peripheral stimulus of RIPC accelerates activation of the intact vagal nerve leading to secretion of ghrelin into circulation via the release of acetylcholine. The present study found that the similarly increased concentrations of UAG after UAG administration also had cardioprotective effects against myocardial ischemia–reperfusion injury, indicating that ghrelin might be one of the humoral factors involved in the cardioprotection of RIPC, which, in turn, is mediated by vagal afferent nerves. While UAG is likely to have cardioprotective effects in RIPC, further studies are needed to reveal the association between modulation of UAG, but not AG, with RIPC and vagal nerve activity.

We observed that $STAT3_{tyr705}$ phosphorylation might be a common cell signaling pathway with both RIPC and UAG. A previous study showed $STAT3_{tyr705}$ has a causal role of the cardioprotection and mitochondrial function [36]. Some previous studies showed that RIPC activates the STAT3 pathway for its cardioprotective effects across species [29, 78]. Similarly, there are some reports describing the cardioprotective effects of UAG [24, 25, 66]. Although the receptor of UAG has not been identified, a previous study found that UAG could protect against doxorubicin-induced cardiomyopathy through growth hormone secretagogue receptor-1a (GHSR1a)-independent pathway, which is a JAK2/STAT3 cell signaling pathway [66]. Other studies showed that UAG and AZP-531, a fragment from native UAG, protect against myocardial ischemia–reperfusion injury in mice, and found that UAG could increase coronary perfusion via NO release and activation of the phosphorylation of extracellular signal-regulated kinase 1/2 and protein kinase B at coronary artery endothelial cells in pigs [24, 25]. We demonstrated that the increased plasma levels of UAG with both RIPC and UAG injection protected against myocardial ischemia–reperfusion injury through activation of the JAK2/STAT3 pathway, as was also previously shown. In another study, Lieder et al. demonstrated that RIPC procedures phosphorylate myocardial $STAT3_{tyr705}$ in pigs with or without vagotomy, and that the spleen plays an important role in the cardioprotective signaling transduction of RIPC in pigs and rats [55]. They found that STAT activation is not sufficient for RIPC cardioprotection but may be required in pigs so well. In fact, their study found that vagotomy with RIPC did not reduce IS in pigs at all. Their results may be attributed to their using large mammals. The vagal tone of rodents differs from that of larger mammals [3, 4, 61]. The heart rate of larger mammals is usually slower than that of smaller animals [82]. Further, it has not been proven whether or not vagotomized rodents with and without RIPC demonstrate activation of the JAK/STAT pathway. Therefore, it may be difficult to compare rodent studies with pig studies. The present study found that $STAT3_{tyr705}$ phosphorylation was associated with the cardioprotection of both RIPC and UAG in rats. Many studies have investigated various cell signaling pathways in RIPC

and UAG in addition to the JAK/STAT pathway, such as the reperfusion injury salvage kinase (RISK) pathway [78]. Further studies are needed to demonstrate whether or not UAG administration activates other cell signaling pathways.

In the present study, we used 0.5 mcg/kg UAG for assessing its cardioprotective effects. However, no report has assessed the cardioprotective effects of a UAG dose of < 100 mcg/kg. Although some studies noted cardioprotective effects of high doses of UAG (100, 1000 mcg/kg and 100 nmol/kg, which is almost equal to 319 mcg/kg), they did not measure the resultant plasma concentrations of UAG [25, 40, 66]. In a preliminary study, we also assessed high UAG doses as in previous studies. Although the dose administered provided cardioprotection against myocardial ischemia–reperfusion injury, plasma levels of UAG after 100 mcg/kg UAG administration were too high to be measured by the ELISA kit (data not shown). Thus, it seems that while the high doses in previous studies have cardioprotective effects on myocardial ischemia–reperfusion injury, the dosage is not physiological and cannot be applied in clinical situation. Based on the results of the preliminary dose study, we investigated the effects of various small doses of UAG (0.25, 0.5, 1, 2, 20 and 30 mcg/kg) on myocardial ischemia–reperfusion injury. We found that all the doses of UAG administered resulted in significant increases in plasma concentrations of UAG compared to baseline, along with reduction in IS except for the group of 0.25 mcg/kg administration, although the ELISA kit was unable to measure plasma levels of UAG after 20, 30 mcg/kg UAG administration (Online resource 3, 4, 5). Therefore, 0.5 mcg/kg UAG administration was adopted in the present study, since the plasma concentration of UAG after 0.5 mcg/kg UAG administration was almost equal to the concentration of UAG after the RIPC procedure within physiological variance. Furthermore, these results suggested that the plasma concentration of UAG resulting from 0.5 mcg/kg UAG administration could simulate the cardioprotective effects of RIPC via activation of the JAK2/STAT3 pathway.

A number of studies reported the humoral factors involved in RIPC, such as, autacoids, hormones, cytokines, chemokines, neuropeptides, amino acids and ribonucleic acid [9, 17, 23, 48, 56, 73, 80]. UAG has characteristics in common with several of the humoral factors in terms of molecular size, hydrophobicity, lyophilization and thermolability [76]. The humoral factors were characterized as having a size of < 12–14 kDa [38, 53, 58, 74, 76]. UAG is a type of peptide hormones and almost 3188 Da in size. Furthermore, a previous study found that circulating AG and UAG are hydrophobic in nature [79]. On the basis of these studies, UAG has the appropriate biophysical characteristics to function as a cardioprotective humoral factor in RIPC.

There is much evidence related to the cardioprotection of remote ischemic conditioning against myocardial ischemia–reperfusion injury in experimental studies [12, 46, 63, 67, 91]. However, the translation from animal experiments to clinical situations for acute myocardial infarction cannot be going well and there are still several additional issues that need to be overcome. This problem can be attributed to the radically different backgrounds of the subjects in each study. While many experimental studies are conducted using healthy young animals with no comorbidities, clinical trials in patients suffering from acute coronary syndrome vary in terms of several factors, such as the patients background, trial protocol, anesthetic conditions, age, sex, comorbidities and comedication, which can attenuate the cardioprotective effects of remote ischemic preconditioning [6, 11, 18, 26, 28, 30, 35, 51, 54, 60, 88]. In addition, the myocardial cell signaling pathways in rodents and humans are different [19, 20, 37, 55, 78]. Although these are the current problems that need to be overcome for translation of RIPC cardioprotection to humans, it is quite possible that elucidation of the unknown mechanisms of remote ischemic conditioning will be clinically beneficial. No experimental studies on the association between RIPC and ghrelin have been investigated. The present study found that the peripheral stimulus of RIPC led to secretion of UAG from the stomach, and that UAG prevented myocardial reperfusion injury through $STAT3_{tyr705}$ activation in rats.

In summary, the present study demonstrated that RIPC procedures increased plasma levels of UAG to a similar level as that resulting from administration of 0.5 mcg/kg UAG administration, both of which exerted their cardioprotective effects in rats through STAT3 phosphorylation. Our data provide new evidence that UAG is one of the key humoral factors in the cardioprotective effects of RIPC.

Study limitation

There are some limitations to this study. First, the validity of our studies might be inadequate because we did not investigate the cardioprotective effects of UAG in the presence of a UAG inhibitor. Future studies using a UAG inhibitor are desired. However, so far, the receptor of UAG has not been identified, and UAG inhibitors are unavailable. Second, we conducted this animal study under anesthesia with three anesthetic agents, midazolam, butorphanol and medetomidine. Previous animal studies were conducted using pentobarbital, sevoflurane, propofol, etc. [7, 8]. Those studies found that the cardioprotective effects of RIPC in rats were abolished by propofol anesthesia, which also inhibited release of the humoral factors of RIPC [7, 8]. These results indicate that the cardioprotective effects might be modulated by the type of anesthetic agents. In future, we should compare plasma levels of UAG under volatile and/or propofol anesthesia. Thirdly, the validation of $STAT3_{tyr705}$

phosphorylation might not be sufficient. The methods used in the present study differed from those of previous studies in terms of the method of sampling myocardial tissues [20, 36, 55, 78]. In this study, we verified the phosphorylation of STAT3 using the discontinuous rat myocardium samplings, obtaining samples just before ischemia and 10 min after reperfusion from separate animals. In previous studies, on the other hand, myocardium samples in both and humans were sequentially obtained just before ischemia and 10 min after reperfusion. Although it is difficult to directly compare the present study with previous studies, our data of Western blots, which using not sequential samplings in rat, were similar to the results of previous studies in pigs with sequential samplings. Finally, additional work is needed to determine the mechanism by which plasma concentration of UAG is increased in RIPC, but not AG, is increased with RIPC.

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Availability of data and material The data are available from the corresponding author on reasonable request.

Compliance with ethical standards

Conflict of interest None of the authors declare any competing interests.

Ethics approval This animal study was approved by the Institutional Animal Care and Use Committee of Sapporo Medical University (No. 17-117,118).

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