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Rivaroxaban, a factor Xa inhibitor, induces the secondary prevention of cardiovascular events after myocardial ischemia reperfusion injury in mice

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ABSTRACT

Objectives: Rivaroxaban has been shown to reduce overall death from cardiovascular causes in patients with recent acute coronary syndrome. Therefore, we evaluated the secondary prevention of cardiovascular events after myocardial ischemia reperfusion injury and its mechanisms in mice.
Methods: After myocardial reperfusion injury, C57BL/6J mice were randomized to receive either no treatment or treatment for 14 days with low and high doses of rivaroxaban. After 7 days, mice were administered tissue factor as a secondary event.
Results: Based on a Kaplan–Meier curve analysis, the high-dose rivaroxaban group showed a significantly higher % survival than the no-treatment group from day 7 (after the administration of tissue factor) to day 14 (at the end of the experimental period). Left ventricular (LV) ejection fraction in both the low- and high-dose rivaroxaban groups improved compared to that in the no-treatment group. Moreover, mRNA levels of interleukin-6 and collagens 1α2 and 3α1 in the LV in the high-dose group were significantly suppressed compared to those in the no-treatment group.
Conclusions: Rivaroxaban improved the survival rate, probably by improving cardiac function through the reduction of inflammatory and fibrotic factors in the LV. This effect may be due to the pleiotropic effects of rivaroxaban beyond its main effect as an anti-coagulant.

1. Introduction

Direct factor Xa inhibitors are currently the mainstream anticoagulant therapy for atrial fibrillation (Af) [1–5]. Various lines of evidence support their safety and effectiveness in clinical trials, and they have also been shown to be effective in the treatment of diseases other than Af [6–12].

In the ATLAS ACS TIMI 51 trial [13], low-dose rivaroxaban was associated with the secondary prevention of cardiovascular events in patients with acute coronary syndrome (ACS), although the mechanism is not yet clear. Various coagulation factors are related to thrombus formation and the inflammatory reaction, and in particular, factor Xa activated protease activation receptors 1 and 2 (PAR-1, 2) and had pro-inflammatory effects [14–20]. PARs are expressed on various cells, including platelets, endothelial cells, myocytes and neurons [21]. PAR-1 induces vasodilation and neutrophilic infiltration, and is related to the progression of the inflammatory reaction [22]. Although PAR-2 mostly has a pro-inflammatory effect, it concurrently has an anti-inflammatory effect [23]. In contrast, rivaroxaban, a factor Xa inhibitor, has an anti-inflammatory effect by inhibiting PARs [24]. We hypothesized that rivaroxaban may induce the secondary prevention of cardiovascular events through an anti-inflammatory effect and examined the effect of rivaroxaban on survival rate and cardio-protection in a model of thrombus formation in mice after myocardial ischemia–reperfusion injury (IRI).

2. Methods

2.1. Animals and drug administration

Male C57BL6/J mice (originally purchased from Japan SLC, Inc.) were used in this study. All experimental procedures conformed to the guidelines for animal experimentation of Fukuoka University. Rivaroxaban was kindly supplied by Bayer HealthCare. After IRI, we randomly divided the animals into three groups: high-dose (1.2 g/kg feed/day) and low-dose rivaroxaban (0.6 g/kg feed/day) groups, and a no-treatment group (control group) (Fig. 1). After 7 days, mice were administered tissue factor as a secondary event. Human tissue factor (TF) (0.3 or 1.5 μg/kg) was injected intravenously into the left retro-orbital venous plexus. The dose was chosen such that at most 10% of control animals survived, and ultimately we selected a dose of 1.5 μg/kg. Mice were given rivaroxaban

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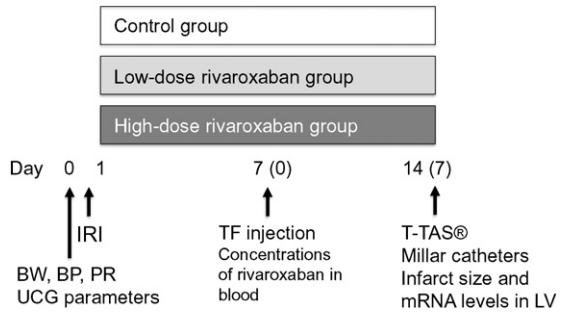

Fig. 1. Study protocol. Parentheses indicate days after tissue factor (TF) injection. BW, body weight; BP, blood pressure; PR, pulse rate; UCG, ultrasound cardiography; IRI, ischemia–reperfusion injury; T-TAS®, Total Thrombus-formation Analysis System; LV, left ventricule.

mixed with feed, since rivaroxaban is metabolized immediately in mice. The administration of rivaroxaban started the day after IRI and continued for 13 days. The mice were housed in a room with a 12 h light/dark cycle and a room temperature of 25 °C.

2.2. An in vivo model of myocardial ischemia–reperfusion injury

A model of coronary occlusion and reperfusion model was prepared in C57BL6 mice using Hutter's method [25]. Mice were anesthetized and ventilated on a Harvard rodent respirator before midline sternotomy. Mice then underwent 30 min of left coronary artery occlusion, and were randomly divided into the three groups described above.

2.3. Blood concentration and the anti-thrombotic effect of rivaroxaban

We confirmed the concentration of rivaroxaban in blood and its anti-thrombotic effects. The blood concentration of rivaroxaban was determined by Shin Nippon Biomedical Laboratories, LTD. The anti-thrombotic effect of rivaroxaban was confirmed by using T-TAS® (Total Thrombus-formation Analysis System, FUJIMORI KOGYO CO., LTD., Japan) [26–28]. T-TAS® is an automated microchip flow-chamber system developed for the quantitative assessment of thrombus formation under variable-flow conditions. With T-TAS®, a "PL-chip" and an "AR-chip" are used to evaluate "platelet-specific" and "comprehensive" thrombus formation, respectively. The PL-chip is used for the quantitative evaluation of platelet thrombus formation. The AR chip is used for the quantitative evaluation of white thrombus formation mediated by the activation of both the coagulation system and platelets. The area under the flow pressure curve (AUC) was computed to assess platelet thrombogenicity inside the microchips. We defined PL-AUC as the AUC for the first 10 min for the PL-chip tested at a flow rate of 18 μL/min. In addition, for the evaluation of white thrombus formation, a whole blood sample with 3.2% sodium citrate was mixed with $CaCl_2$ (20 μl) just before measurement. The mixture, which had a volume of 480 μl, was applied to the AR-chip at a flow rate of 10 μl/min, and the initial wall shear rate was estimated to be 600 s^{-1}. We defined AR-AUC as the AUC for the first 30 min.

2.4. Determination of cardiac function at baseline by ultrasound cardiography (UCG)

We used UCG (TOSHIBA CORP., Japan) to measure interventricular septal thickness dimension (IVSTd), left ventricular diastolic dimension in diastole (LVDd), left ventricular posterior wall dimension (LVPWd), left ventricular systolic dimension (LVSd), left ventricular ejection fraction (LVEF), and fractional shortening (FS) by the M-Mode method just before IRI.

2.5. Millar catheters

We used Millar's SPR-839 Mikro-Tip® ultra-miniature PV loop catheter to monitor high-fidelity cardiovascular pressures and measured various parameters of cardiac function at 14 days after IRI. We used open-chest surgical procedures, and approached from the apex of the heart.

2.6. Evans blue staining

At 14 days after IRI, the ligature around the coronary artery was retied and 1 ml of 2% Evans blue dye was injected into the left ventricular cavity. The dye was circulated and uniformly distributed except in the portion of the heart that had been previously perfused by the occluded coronary artery (area-at-risk, AAR). The heart was quickly excised and sliced into five equal parts along its long axis. Slices were incubated individually in 1% TTC in phosphate buffer at 37 °C for 10 min, and photographed with a digital camera (Olympus, Japan). The Evans blue-stained area (area-not-at-risk, ANAR), TTC-stained area (area at risk: AAR), and TTC staining-negative area (infarct area: IA) were measured digitally using Image J software. The myocardial infarct size was expressed as a percentage of the total AAR [29,30].

2.7. Reverse transcription, real-time polymerase chain reaction

Total RNA was isolated from LV slices using a Handy Micro-homogenizer (Microtec LTD., Tokyo) in combination with TRIzol Reagent (Thermo Fisher Scientific, Waltham, MA) at 14 days after IRI. Reverse transcription was performed using a QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany) from 1 mg of extracted total RNA. Quantitative real-time PCR (qPCR) was performed on a 7500 Fast Real-Time PCR System (Applied Biosystems, Waltham, MA) using gene-specific primers and Power SYBR Green PCR Master Mix (Qiagen). Initial denaturation at 95 °C for 600 s was followed by 40 cycles of denaturation at 95 °C for 10 s, annealing at 60 °C for 20 s, and elongation at 72 °C for 20 s. Data are expressed in arbitrary units that were normalized by the results for β-actin. Expression analyses were carried out according to the ΔΔCT method. The amplification specificity of PCR products was confirmed by a melting curve analysis and agarose gel electrophoresis. Atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), interleukin-6 (IL-6), monocyte chemoattractant protein-1 (MCP-1), collagen 1α2, collagen 3α1, protease activation receptor-2 (PAR-2), PAR-3 and PAR-4 were investigated. The primers used were forward primer 5′-GGGGGTAGGATTGACAGGAT-3′ and reverse primer 5′-ACACACCACA AGGGCTTAGG-3′ for mouse ANP, forward primer 5′-TCCTAGCCAGTCTCCAGAGC-3′ and reverse primer 5′-CCTTGGTCCTTCAAGAGCTG-3′ for mouse BNP, forward primer 5′- CACAAGTCCGGAGAGGAGAC −3′ and reverse primer 5′- CAGAATTGCCATTGCACAAC −3′ for mouse IL-6, forward primer 5′-AGCACCAGCCAACTCTCACT −3′ and reverse primer 5′- GGCGTTAACTGCATCTGGCT −3′ for mouse MCP-1, forward primer 5′-CCCCGGGACT CCTGGACTT-3′ and reverse primer 5′-GCTCCGACACGCCCTCTCTC-3′ for mouse collagen 1α2, forward primer 5′-TTGATGTGCAGCTGGCATTC-3′ and reverse primer 5′- GCCACT GGCCTGATCCATAT-3′ for mouse collagen 3α1, forward primer 5′-CACCTGGCAAGAAG GCTAAG-3′ and reverse primer 5′- CCCAGGGTTACTGACGCTAA-3′ for mouse PAR-2, forward primer 5′- TCAATGGCAACAACTGGGTA −3′ and reverse primer 5′- AAAACCATGA CCCACACCAT −3′ for mouse PAR-3, forward primer 5′- GCAGACCTTCCGATTAGCTG-3′ and reverse primer 5′- AGGGCTCGGGTTTGAATAGT-3′ for mouse PAR-4, and forward primer 5′- CCACACCCGCCACCAGTTCG −3′ and reverse primer 5′- TACAGCCCGGGGAG CATCGT −3′ for mouse β actin.

2.8. Data analysis

All values in the text and figures are presented as means ± standard errors (S.E.) of n independent experiments. All analyses were performed using PRIZM 6 software (GraphPad Prism, San Diego, CA). All data were analyzed by one-way ANOVA followed by the Bonferroni correction for post hoc t-tests (GraphPad Prism). Kaplan–Meier survival curves were also analyzed by PRIZM 6. Probabilities of 0.05 or less were considered to be statistically significant.

3. Results

3.1. Determination of the doses of rivaroxaban

Based on the effects of rivaroxaban, the doses of rivaroxaban in the high- and low-dose groups were set at 1.2 and 0.6 g/kg feed/day. The effects of rivaroxaban were evaluated in terms of the concentration of rivaroxaban in blood and the results of T-TAS® (Fig. 2). Concentrations of rivaroxaban in the low-dose and high-dose groups after 7 days were 0.34 ± 0.20 and 0.79 ± 0.16 μg/ml, respectively (low-dose vs. high-dose, p = 0.02) (Fig. 2A). The effects of rivaroxaban using T-TAS® after 14 days are shown in Fig. 2 BC. AR-AUC, but not PL-AUC, in the low-dose group was significantly lower than that in the control group. In addition, AR-AUC in the high-dose group was significantly lower than that in the low-dose group. Thus, rivaroxaban significantly prevented thrombus formation in the high-dose group.

3.2. Baseline characteristics in the low-dose and high-dose rivaroxaban and control groups

Table 1 shows body weight (BW), blood pressure (BP), pulse rate (PR), and various other parameters determined using UCG at baseline just before IRI. There were no significant differences in the baseline characteristics including age, BW, BP, PR and various UCG parameters among the control and low-dose and high-dose rivaroxaban groups.

3.3. Kaplan–Meier survival curves

We used the Kaplan–Meier method with the log-rank test to examine survival (Fig. 3). There was a marginally significant difference in survival curves between the control and high-dose groups ($p = 0.06$) (Fig. 3A). There was no significant difference before the injection of TF (Fig.

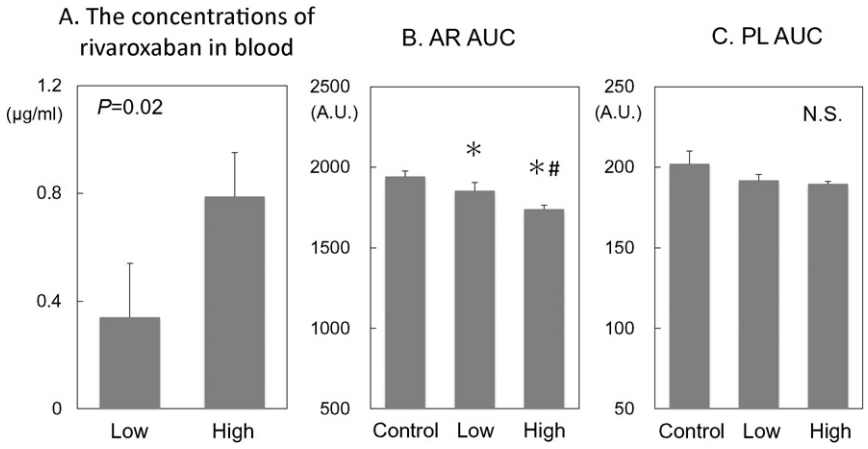

Fig. 2. Effects of rivaroxaban evaluated in terms of the concentration of rivaroxaban in blood (A) and the results of T-TAS® (B and C). N.S., not significant; AU, arbitrary units. *$P < 0.05$ vs. control group. #$P < 0.05$ vs. low-dose group.

3B). When we evaluated the survival curves just before and after the injection of tissue factor, there was a significant difference in the survival rate after injection between the control and high-dose groups ($p < 0.05$) (Fig. 3C).

3.4. Infarct size in myocardium, cardiac function and BP in the low-dose and high-dose rivaroxaban and control groups

The myocardial infarct size was determined by the Evans blue/TTC double-staining method. There were no significant differences among the 3 groups (Fig. 4AB). There were also no differences in systolic BP (SBP), mean BP (MBP) or diastolic (DBP) among the 3 groups (Fig. 4C-E). We assessed cardiac function at the end of the experimental period using a Millar catheter (Fig. 4F–H). LVEF was significantly improved in both the high-dose and low-dose rivaroxaban groups [high-dose group (47 ± 16%) vs. control group (27 ± 10%), p = 0.009; low-dose group (39 ± 11%) vs. control group, p = 0.039]. Since LVEF in sham-operated mice was 84 ± 9%, LVEF in the control group was significantly decreased due to myocardial IRI. Stroke volume (SV) in the high-dose group was significantly improved compared to that in the control group, whereas cardiac output (CO) in the high-dose group was higher than that in the control group, but not significantly.

Table 1
Baseline characteristics in the control, low-dose and high-dose groups.

	Control	Low-dose	High-dose
Number of mice	32	21	20
Age (weeks)	10.0 ± 1.2	10.6 ± 1.1	10.4 ± 0.9
BW (g)	25.4 ± 1.1	25.9 ± 1.2	25.7 ± 1.4
BP (mmHg)			
Systolic	82.1 ± 14.5	86.6 ± 13.4	82.4 ± 11.7
Diastolic	29.5 ± 7.5	35.7 ± 17.5	32.1 ± 6.7
Mean	44.1 ± 9.0	51.8 ± 14.6	48.4 ± 9.3
PR (/min)	525.1 ± 34.7	511.0 ± 57.7	495.0 ± 52.3
IVSTd (mm)	0.76 ± 0.05	0.73 ± 0.06	0.75 ± 0.07
LVDd (mm)	3.90 ± 0.42	3.77 ± 0.58	3.66 ± 0.44
LVPWd (mm)	0.74 ± 0.13	0.75 ± 0.12	0.76 ± 0.12
LVDs (mm)	2.36 ± 0.35	2.26 ± 0.41	2.14 ± 0.36
CO (L/min)	0.058 ± 0.02	0.055 ± 0.02	0.047 ± 0.02
LVEF (%)	76 ± 8	77 ± 6	78 ± 6
LVFS (%)	40 ± 7	40 ± 6	41 ± 6

BW, body weight; BP blood pressure; PR, pulse rate; IVSTd, interventricular septum thickness diameter; LVDd, left ventricular internal dimension in diastole; LVPWd, left ventricular posterior wall thickness diameter; LVDs, left ventricular internal dimension in systole; CO, cardiac output; LVEF, left ventricular ejection fraction; LVFS, left ventricular fractional shortening.

3.5. mRNA levels of markers of inflammation, fibrosis and PARs

We also examined the effects of rivaroxaban on mRNA levels of various factors in the LV using RT-PCR (Fig. 5). Rivaroxaban significantly reduced the mRNA levels of the inflammatory factor IL-6 (Fig. 5C). Rivaroxaban also significantly suppressed the expression of mRNA for fibrotic factors including collagens 1α2 and 3α1 (Figure 5EF). In addition, we determined the effects of rivaroxaban on mRNA levels of PARs in LV (Figure 5G-I). Rivaroxaban significantly reduced the mRNA level of PAR-2 in both the low-dose and high-dose groups and the mRNA level of PAR-4 in the high-dose group.

4. Discussion

The main finding in this study was that the survival rate after the injection of TF in the high-dose rivaroxaban group was significantly higher than that in the control group. In addition, the high-dose group showed significantly improved cardiac function. We also examined the effects of rivaroxaban on mRNA levels of various factors and found that rivaroxaban improved cardiac function probably due to anti-inflammatory and anti-fibrotic effects.

Rivaroxaban reduced the expression levels of mRNA of the inflammatory factor IL-6, and also reduced the mRNA levels of collagens 1α2 and 3α1 in LV. Previous reports indicated that rivaroxaban has anti-inflammatory and anti-fibrotic effects by inhibiting PAR [14–20]. Furthermore, cardiac hypertrophy was observed in transgenic mice that overexpressed cardiomyocyte-specific PAR-2, and cardiac remodeling after myocardial infarction was controlled in PAR-2 knockout mice [31]. Therefore, we examined the effects of rivaroxaban on mRNA levels of PARs in the LV using RT-PCR. Rivaroxaban reduced the expression levels of PAR-2 in both the low-dose and high-dose groups and the level of PAR-4 in the high-dose group in LV. Thus, rivaroxaban may help to prevent cardiac remodeling by reducing the inflammation and fibrosis associated with a decrease in the expression levels of PARs in LV independent of its anticoagulant effect, and subsequently may improve cardiac function.

Next, we elucidated that the improved cardiac function helped to improve the survival rate, in addition to protecting against total-body embolism. When we removed the hearts from mice that had died before TF administration, some showed myocardial rupture. Since these mice that died with myocardial rupture all died within a few days after reperfusion injury, and since myocardial rupture is a condition of irreversible and serious tissue damage, rivaroxaban could not prevent myocardial rupture and did not improve the survival rate before TF administration.

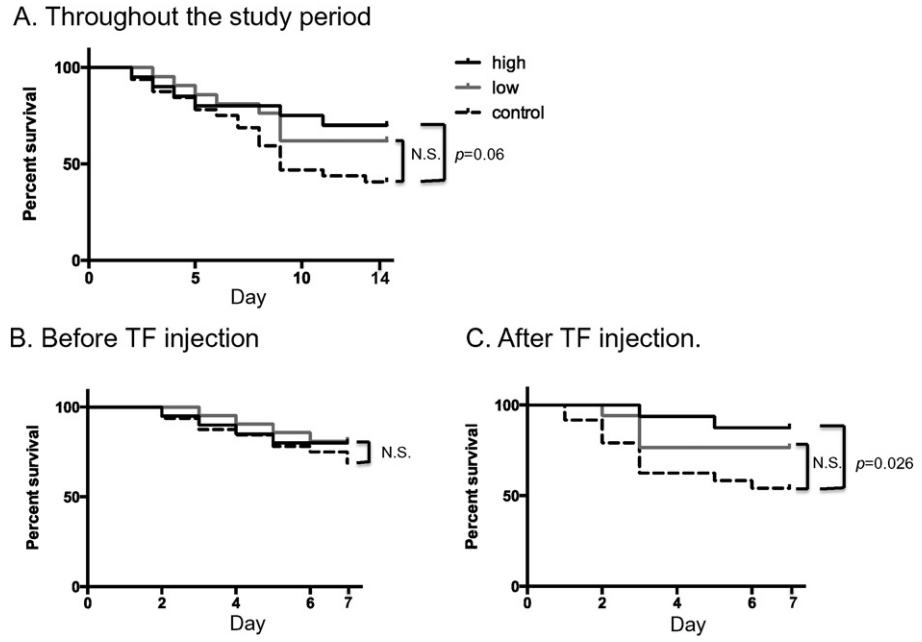

Fig. 3. Kaplan–Meier survival curves throughout the study period (A), and before (B) and after (C) tissue factor injection. N.S., not significant.

On the other hand, the survival rate after TF injection in the high-dose group was improved. PARs are mainly expressed on platelets in addition to myocytes, and activate platelets and cause thrombosis. Rivaroxaban blocked the activation of PAR, and this improved the survival rate by inhibiting new thrombus events [20,24]. After TF administration, the main cause of death may be total-body embolism because death in these cases was mainly due to pulmonary embolism [32,33]. Since rivaroxaban improved cardiac function due to the reduction of expression levels of PARs in LV, it may also help to prevent cardiac dysfunction by TF-induced pulmonary embolism, and subsequently improves the survival rate.

5. Conclusion

Rivaroxaban contributed to the secondary prevention of cardiovascular events after IRI, since rivaroxaban conferred cardio-protection, including the improvement of cardiac function, through anti-inflammatory and anti-fibrotic effects.

Conflict(s) of interest/disclosure(s)

K.S. and S.M. have received grants from Bayer Yakuhin Ltd. and lecture honoraria from Takeda Co. Ltd. K.S. is a Chief Director and S.M. is

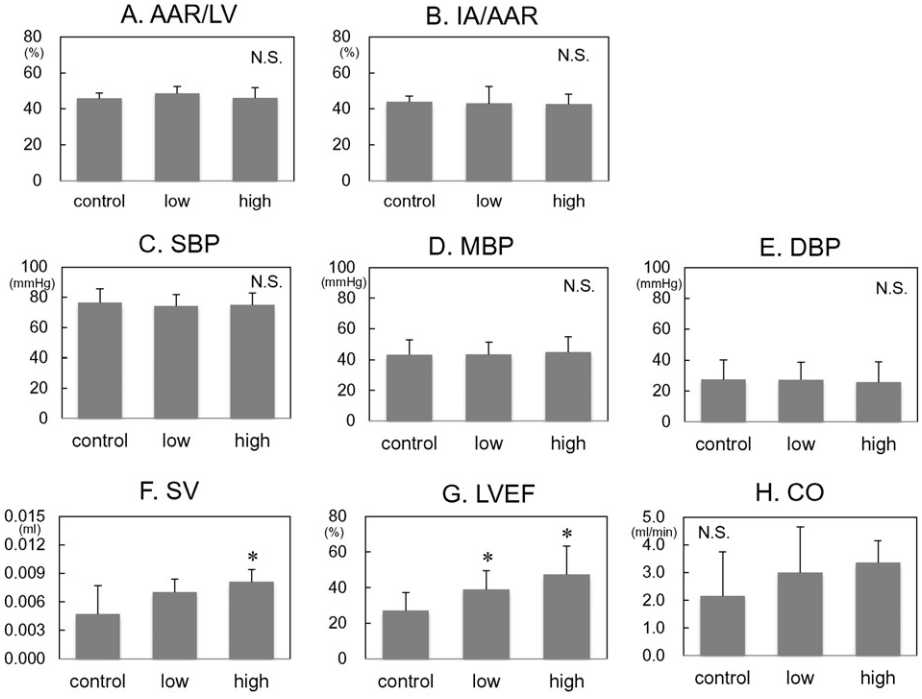

Fig. 4. The myocardial infarct size by Evans Blue staining and cardiac function after 2 weeks. N.S., not significant; AAR, area at risk; LV, left ventricle; IA, infarct area; SBP, systolic blood pressure; MBP, mean blood pressure; DBP, diastolic blood pressure; SV, stroke volume; LVEF, left ventricular ejection fraction; CO, cardiac output. *$P < 0.05$ vs. control group.

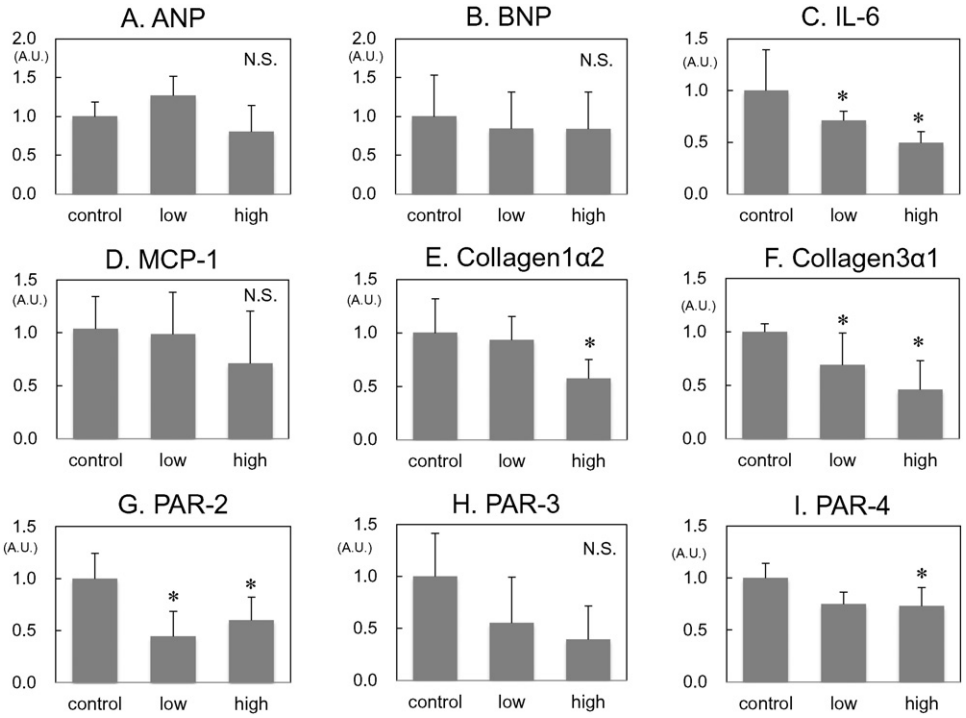

Fig. 5. mRNA levels of monocyte chemoattractant protein-1 (MCP-1) (A), interleukin-6 (IL-6) (B), atrial natriuretic peptide (ANP) (C), brain NP (BNP) (D), collagen 1α2 (E), collagen 3α1 (F), and protease activation receptors (PAR)-2,3 and 4 (G, H and I) in the left ventricle. mRNA levels were determined by quantitative real-time PCR and are expressed relative to β-actin levels. The basal expression relative to the respective value in the control group was considered to be 1.0. A.U., arbitrary units; N.S., not significant. $^{*}P < 0.05$ vs. control group.

a Director of NPO Clinical and Applied Science, Fukuoka, Japan. S.M.'s spouse is an employee of Bayer Yakuhin Ltd. K.S. has an Endowed "Department of Molecular Cardiovascular Therapeutics" supported by MSD, Co. Ltd. S.M. and Y.U. belong to the Department of Molecular Cardiovascular Therapeutics, which is supported by MSD, Co. Ltd.

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