Title: Postischemic infusion of sivelestat sodium hydrate, a selective neutrophil elastase inhibitor, protects against myocardial stunning in swine

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Abstract

Purpose. It seems controversial whether or not neutrophil elastase inhibitors are effective in attenuating the myocardial ischemia/reperfusion injury. We thus investigated possible protective effects of sivelestat, a neutrophil elastase inhibitor, against myocardial stunning-i.e., prolonged myocardial dysfunction following a brief episode of ischemia.

Methods. Swine were divided into control group (group C), low-dose sivelestat group (group L), and high-dose sivelestat group (group H) (n=7 for each group). All the swine were subjected to myocardial ischemia through ligation of the left anterior descending coronary artery (LAD) for 12-min, followed by 90-min reperfusion. Sivelestat was infused intracoronally at concentrations of 6 and 60 mg/ml throughout the reperfusion period in the group L and H, respectively, while saline in the group C. Heart rate (HR), left ventricular developed pressure (LVdP), maximum rate of LVdP (LVdP/dtmax), LV end-diastolic pressure (LVEDP), percentage of segment shortening (%SS, an index of regional myocardial contractility), and coronary venous interleukin-6 concentration in LAD perfusion area were measured before ischemic induction and during reperfusion.

Results. The ischemia-reperfusion insult did not cause any significant changes in the HR, LVdP, LVdP/dtmax, and LVEDP in all groups. However, it significantly decreased %SS in the LAD perfusion area, while increased the interleukin-6 concentration in the group C. Those changes in %SS and the interleukin-6 concentration were both greatly attenuated, but not prevented, in the group L and group H.

Conclusion. Sivelestat presumably attenuates myocardial contractile dysfunction due to myocardial stunning by inhibiting neutrophil-derived elastase and thereby suppressing the production of interleukin-6 in activated neutrophils.

(250 words)

Introduction

Neutrophil elastase is capable of degrading many structural proteins, notably elastin, collagens and fibrinogen, and is thought to participate in cardiovascular damage encountered in various pathologies. Neutrophil elastase is also known to induce production of cytokines, such as interleukin (IL)-6, which decreases cardiac contractility via a nitric oxide-dependent pathway [1]. Neutrophil elastase has been shown to be released in early stages of postischemic myocardial reperfusion in animal models [2], as well as during unstable angina attacks after myocardial infarction or cardiopulmonary bypass surgery in humans [3]. At this time, little evidence is available regarding the efficacy of neutrophil elastase inhibitors against the myocardial ischemia/reperfusion injury [4].

Myocardial stunning is defined as prolonged reversible contractile dysfunction following a brief ischemic episode that does not result in necrosis [5]. It occurs associated with unstable angina attacks [6], exercise-induced ischemia [7], percutaneous transluminal coronary angioplasty [8], and open heart surgery [9]. The mechanisms behind myocardial stunning could be, in part, different from those behind myocardial infarction [10]. Noutrophils

have been proposed to play a key role in mediating the ischemia/reperfusion injury [11, 12], however their precise role in the development of myocardial stunning, a type of the ischemia/reperfusion injury, has not yet been clarified. In addition, it seems controversial whether neutrophil elastase inhibitors are effective in attenuating the myocardial stunning [13, 14].

Sivelestat sodium hydrate (sivelestat), a selective neutrophil elastase inhibitor [15], has been clinically used for acute lung injury and adult respiratory distress syndrome. Recent animal studies have shown that sivelestat reduces the ischemia-reperfusion injury associated with liver [16], lung [17], or heart [18] transplantation.

In this study, utilizing anesthetized swine undergoing ischemia/reperfusion, we investigated whether sivelestat, infused intracoronarily during the reperfusion period, is effective in attenuating the myocardial stunning, as well as in reducing proinflammatory cytokine production in the ischemic area.

Materials and methods

Surgical procedures

All experimental procedures used in this investigation were reviewed and approved by the Institutional Animal Care Committee. Twenty-seven swine (20-35 kg) of either sex were sedated with ketamine hydrochloride, 20 mg/kg, intramuscularly. Swine were anesthetized with α-chloralose, 100 mg/kg, and fentanyl, 10 μg/kg, intravenously, followed by continuous infusion of α-chloralose, 10 $mg\text{-}kg^{-1}\text{-}h^{-1}$, and fentanyl, 5 $\mu g\text{-}kg^{-1}\text{-}h^{-1}$, throughout the study period. Through a midline cervical incision, the trachea was intubated for connection to a Harvard respiratory pump (Harvard Apparatus Co., South Natick, MA). Mechanical ventilation was facilitated by an intermittent IV infusion of vecuronium, 0.2 mg/kg. Tidal volume, respiratory rate, and inspired oxygen concentration were adjusted to maintain the arterial carbon dioxide tension ($PaCO_2$) between 35 and 40 mmHg, and the arterial oxygen tension (PaO_2) between 100 and 300 mmHg. End-tidal CO_2 concentration was continuously monitored by using a gas analyzer (Capnomac Ultima, Datex, Helsinki, Finland). Lactated Ringer's solution was infused at a rate of 5 $ml\text{-}kg^{-1}\text{-}h^{-1}$. Sodium bicarbonate and blood glucose concentrations were

measured before and during ischemia and maintained within physiological range throughout the study period. The esophageal temperature was maintained at between 36°C and 37°C throughout the study period by using a warmer blanket and a heating lamp. A heparin-filled catheter was inserted into the right carotid vein to administer fluid and drugs. A standard peripheral lead electrocardiogram was monitored continuously. A medial sternotomy was performed and the pericardium was opened, exposing the heart. Systemic anticoagulation was achieved with intravenous sodium heparin, 750 U/kg, followed by a continuous infusion, 250 $U\text{-}kg^{-1}\text{-}h^{-1}$. The left anterior descending coronary artery (LAD) distal to the first diagonal branch was cannulated with a stainless-steel cannula and perfused with blood from the left carotid artery through an extracorporeal circuit. Coronary perfusion pressure was measured from the sidearm of the circuit, using a pressure transducer-tipped catheter (PC500; Millar Instruments, Huston, TX, USA), and coronary blood flow (CBF) of the perfusion area of LAD was measured with an ultrasonic flow probe (ADP17; Crystal Biotech, Hopkinton, MA) attached at the circuit. The circuit also contained a distal infusion port for drug administration. A 22-gauge catheter was inserted into epicardial vein

at the perfusion area of LAD to allow coronary venous blood sampling. The venous cannula was allowed to drain freely into a beaker to prevent venous stagnation. This venous blood was returned intermittently to the swine to maintain isovolemic conditions. A pressure transducer-tipped catheter was inserted into left ventricular (LV) chamber through an incision in the apex for continuous recording of LV pressure (LVP). The peak rate of increase in LVP ($LVdP/dt_{max}$) was determined by electric differentiation of the LV pressure waveform. A pair of ultrasonic segment length transducers was implanted in the subendocardium of the perfusion area of LAD to measure changes in regional contractile function (percentage segment shortening [%SS]). Segment length was monitored by ultrasonic amplifiers (VF-1; Crystal Biotech, Hopkinton, MA). The end-systolic segment length (ESL) was determined 10 ms before maximum negative LVdp/dt, and the end-diastolic segment length (EDL) was determined 10 ms before the LVdP/dt first exceeded 140 mmHg/s (immediately before the onset of LV isovolemic contraction). %SS was calculated using the formula: $\%SS = (EDL - ESL) \times 100 \times 1/EDL$. All hemodynamic data were continuously monitored on a polygraph and digitized via a computer interfaced with an

analog-to-digital converter (HEM; Physio-Tech, Tokyo, Japan).

Experimental protocols

Figure 1 shows the experimental time course. Thirty minutes after the instrumentation was completed, baseline systemic and coronary hemodynamics and %SS were recorded. Swine were randomly allocated to one of three groups. Group L and H (n=7 in each group) received intracoronary infusion of sivelestat at concentration of 6 and 60 μg/ml, respectively, from the beginning of reperfusion until the end of experiment. Group C (n=7) received saline in place of sivelestat. The intracoronary infusion rate of sivelestat (μg /min) was determined using the formula: the targeted coronary blood concentration, 6 or 60 μg/ml, × prevailing CBF rate (ml/min). The concentration of 6 μg/ml corresponded to clinically applied concentration for acute lung injury (ALI) [19].

All swine were subjected to 12-min ischemia with complete occlusion of the extracorporeal circuit followed by a 90-min reperfusion. Systemic and coronary hemodynamics and myocardial contractile function were monitored continuously throughout the experiment and recorded at the time points illustrated in figure 1 (P_0, baseline; R_0, just before reperfusion; R_5, R_{30}, R_{60},

and R_{90}, 5, 30, 60 and 90 min after reperfusion). Coronary venous blood samples of the LAD perfusion area were collected at P_0 and R_{90}, and the coronary venous IL-6 concentration was measured by ELISA method.

All swine received intravenous lidocaine, 2 mg/kg, at 1 min before reperfusion. If five or more premature ventricular contractions per minute or multifocal premature ventricular contractions were observed after reperfusion, intravenous lidocaine, 1 mg/kg, was administered and repeatedly given if necessary. Swine with continuous ventricular fibrillation or ventricular tachycardia after reperfusion were excluded from the study.

Statistical analysis

All data are expressed as mean±SD. Statistical analysis was performed with Stat View (Abacus Concepts, Berkeley, CA, USA) and Super ANOVA (Abacus Concepts, Berkeley, CA, USA). Hemodynamic data and %SS between groups were analyzed with one-way factorial analysis of variance (ANOVA) and Scheffe's test, and those within groups were analyzed with one-way repeated measures ANOVA and contrast analysis. Equal variances of coronary venous IL-6 concentrations between and within groups were analyzed with Bartlett's test. Coronary venous IL-6 concentrations between groups at

baseline were analyzed with one-way factorial ANOVA and Scheffe's test, and those at 90 min after reperfusion were analyzed with Kruskal-Wallis test and Scheffe's test. IL-6 concentrations within groups were analyzed with paired t-test. P values<0.05 were considered statistically significant.

Results

There were no significant differences in weight or sex among groups. Arterial blood gas values and blood glucose were maintained within physiological range in all swine throughout the study period (Table 1).

The ischemia/reperfusion insult did not cause any significant changes in heart rate, LVdP, LVdP/dtmax, and LVEDP in all groups (Table 2), suggesting that the insult did not cause any significant changes in overall cardiac function. In addition, no significant differences were observed in these variables at any time point among the three groups (Table 2).

Complete occlusion of the extracorporeal circuit (i.e., induction of ischemia), as expected, resulted in complete cessation of the CBF in the LAD area in all groups (Table 2). In addition, in response to the release of occlusion (i.e., reperfusion), the CBF acutely increased to the level much higher (~almost twice) than the preischemic level in all groups (see the values at R5 in Table 2), suggesting the occurrence of expected reperfusion hyperemia. Thereafter, the CBF gradually decreased to the preischemic level in all groups (Table 2). No significant differences were observed in the CBF at any time point among the three groups (Table 2). Sivelestat

administration did not influence systemic or coronary hemodynamics throughout the time course (Table 2).

The induction of ischemia resulted in great reductions of the %SS, an index of contractile function of the LAD-perfused area, to the minus level (Table 2 & Figure 2), suggesting the occurrence of ventricular bulging. In response to the reperfusion, despite the recovery of CBF to the preischemic level, the %SS did not recover to the preischemic level in all groups (Table 2 & Figure 2). Specifically, it recovered only by 30-49% 5 min after the reperfusion in all groups; no significant differences were observed in the %SS value immediately after the reperfusion among the three groups (see the R5 values in Table 2 & Figure 2). During the 90-min reperfusion period, the %SS did not further recover in the group C, but gradually recovered in the group L and group H (Table 2 & Figure 2). However, no significant differences were observed in the %SS at any time point between the group L and group H (Table 2 & Figure 2).

The IL-6 concentration in the venous blood returning from the LAD-perfused area (subjected to ischemia) greatly (about 5-fold) increased 90 min after reperfusion in the group C, while it only slightly, but

significantly, increased in the group L and group H (Figure 3). However, no significant difference was observed in the IL-6 concentration 90 min after reperfusion between the group L and group H (Figure 3).

Discussions

Myocardial stunning is a type of ischemia/reperfusion injury characterized by reversibly impaired postischemic myocardial contractile function, in which perfusion is normal or close to normal. This contractile dysfunction would last hours to days [5]. This ischemia/reperfusion injury could occur after cardiac surgery, cardiopulmonary resuscitation or percutaneous transluminal coronary angioplasty. Some perioperative agents such as sevoflurane [20] or milrinone [21] have been suggested to serve as a protectant against myocardial ischemic-reperfusion injury. However, their clinical effectiveness has not yet been established.

This study for the first time demonstrates that a neutrophil elastase inhibitor administered in postischemic period could be effective against myocardial stunning. Specifically, in this study, sivelestat intracoronarily administered immediately after reperfusion attenuated both the decrease in myocardial contractility (i.e., %SS) and increase in coronary venous IL-6 concentration in the reperfused area. Thus, sivelestat administered immediately after reperfusion seems to exert cardioprotective effects against the ischemia/reperfusion-induced myocardial stunning, at least in part by

suppressing the proinflammatory cytokine production. Although we did not confirm reversibility of the contractile dysfunction (decreases in %SS), the short period of preceding ischemia (i.e., 12 min) and the observed mismatch between CBF and contractility were both consistent with myocardial stunning. Our experimental model might be accordance with well established model of myocardial stunning in previous report [22].

Neutrophils degranulate to release proteases, collagenases, lipoxygenases, phospholipases, and myeloperoxidase. The serine protease, elastase, is a major contributor to neutrophil-mediated damage, and hydrolyzes the extracellular matrix components elastin, fibronectin and collagen types III and IV [23]. Neutrophil elastase induces production of cytokines which decreases cardiac contractility via a nitric oxide-dependent pathway [1], and produces toxic mediators such as reactive oxygen species (ROS) [24]. ROS is strongly implicated in the pathogenesis of myocardial stunning, necrosis, apoptosis and vascular dysfunction. Ohta et al. [25] reported that elastase inhibition can significantly improve the ventricular function and remodeling in an infarct model. However, the contribution of neutrophils to myocardial stunning remains controversial [13, 14].

The present results suggest a possibility that an elastase inhibitor administered during the reperfusion period may improve contractility of the reperfused myocardium by reducing the production of proinflammatory cytokines. Thus, the neutrophil elastase would be significantly involved in the development of myocardial stunning. Ueno et al. [26] demonstrated that sivelestat administered before reperfusion attenuated the elevation of neutrophil elastase and proinflammatory cytokines without depression of leukocyte or neutrophil count in canine heart transplantation model. In the present study, sivelestat significantly suppressed the elevation of IL-6 at 90 min after reperfusion. IL-6 is one of proinflammatory cytokines which is not constitutively expressed in the normal heart. Upregulation and production of this cytokine represent an intrinsic or an innate stress response against myocardial injury. IL-6 could accelerate myocardial necrosis and apoptosis leading to contractile dysfunction [27]. Kukielka et al. [28] reported that cytokines not only induce ROS production but also are themselves induced by ROS. Haga et al. [29] demonstrated that sivelestat at clinically available concentrations can inhibit proinflammatory cytokine production in isolated human monocytes. Further examination is needed to clarify whether the

suppression of IL-6 production could result from inhibition of neutrophil elastase activation or inhibition of proinflammatory cytokine release from monocytes.

In the present study, a higher dose of sivelestat did not exert greater effects compared with a lower dose. The clinically applied concentration for ALI of sivelestat (a lower dose: 6 μg/ml) would be sufficient to inhibit neutrophil elastase activity in the reperfused myocardium.

The myocardial global contractility would be influenced by the size of ischemia/reperfusion area. Thus LVdP/dtmax in reperfusion period showed no significant difference between groups in spite of significant difference in %SS. %SS has gained broad acceptance as an index of regional cardiac function because it can be easily applied in experiments and because it measures parameters that are intuitively reasonable [30]. However, it was reported that %SS is insensitive when measuring high contractility and overly sensitive when measuring low contractility compared to the slope of end-systolic pressure volume relationship (Emax) [31]. Although Emax would be superior to %SS as an index of cardiac function in the global ischemia model, the ischemic region in the present study might be

comparatively small and LVdP/dtmax showed no significant change. Thus we applied %SS to the index of regional cardiac function.

Zaugg et al. suggested that the choice of background anesthesia may play a role in cardiac protection in both experimental and clinical medicine [32]. We used fentanyl and ketamine as background anesthesia in the present study. Fentanyl was previously reported to reduce postischemic infarct size but not affect functional recovery of stunned myocardium [33]. Ketamine was previously reported to block adenosine triphosphate-sensitive potassium channel opening which plays a crucial role in cardioprotection against ischemia/reperfusion injury [32]. Therefore we could not completely deny the possible contribution of background anesthesia to the cardioprotection observed in the present study.

In conclusion, sivelestat intracoronarily administered throughout the reperfusion period exerts cardioprotective effects. The mechanism of this cardioprotective effect would involve the suppression of proinflammatory cytokine production.

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Figure legends

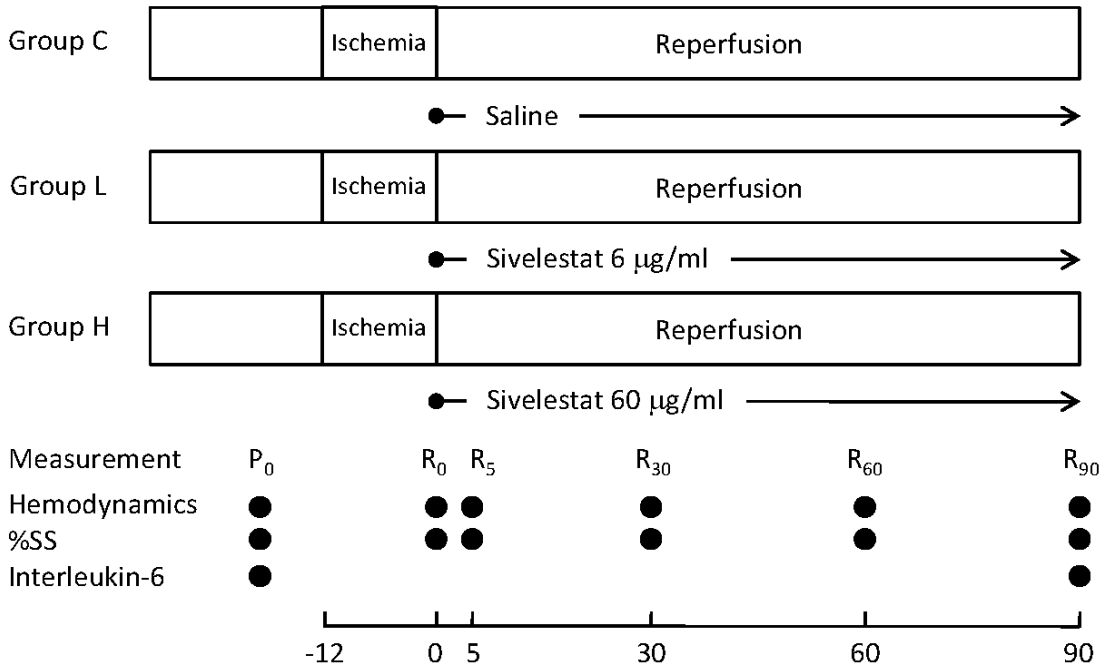

Figure 1. Experimental time course. All swine were subjected to 12-min ischemia followed by 90-min reperfusion. Group L or H (n=7 in each group) received intracoronally sivelestat at the arterial concentration of 6 or 60 μg/ml starting just after reperfusion until the end of experiment. Group C (n=7) received saline in place of sivelestat. Hemodynamic and percentage segment shortening (%SS) measurements (black triangle), and coronary venous blood sampling for the measurement of interleukin-6 concentrations (black circle) were performed at the time indicated in the figure. P_0, baseline; R_0, just before reperfusion; R_5, R_{30}, R_{60} and R_{90}, 5, 30, 60 and 90 min after reperfusion

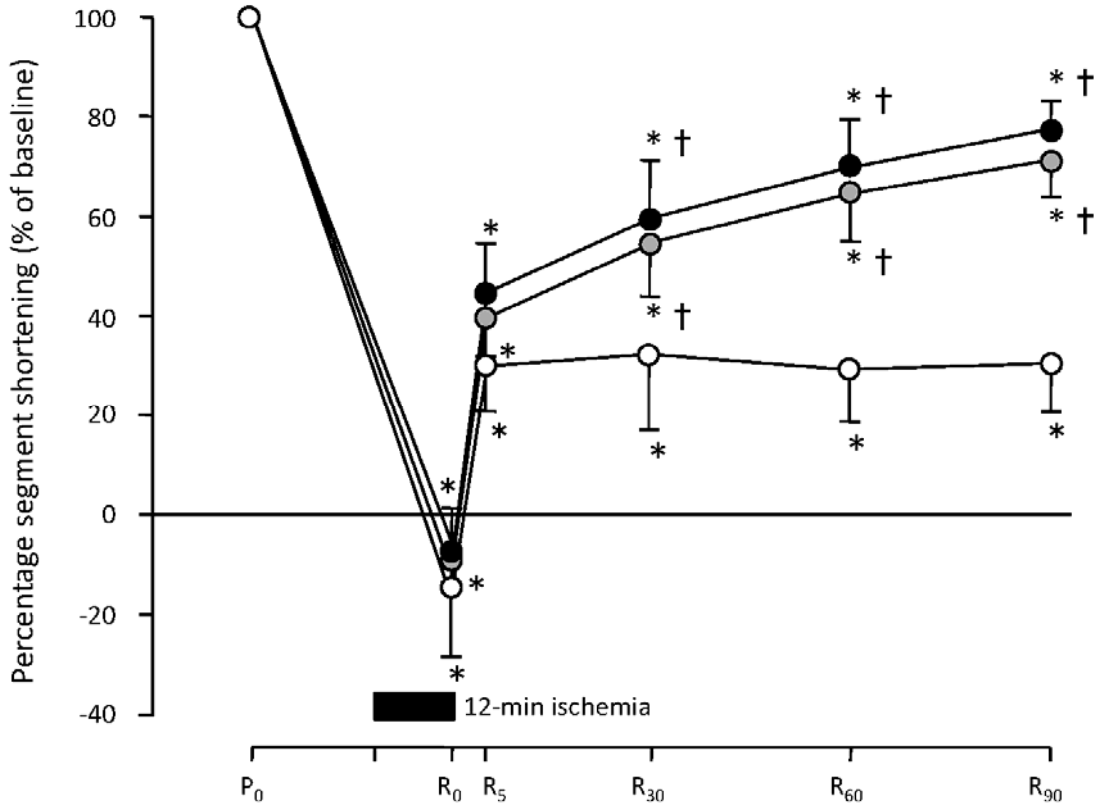

Figure 2. Recovery of segment shortening. Values are mean±SD. N=7 in each group. *: $p<0.05$ vs. P_0; †: $p<0.05$ vs. group C. White circle, group C; gray circle, group L; black circle, group H; P_0, baseline; R_0, just before reperfusion; R_5, R_{30}, R_{60} and R_{90}, 5, 30, 60 and 90 min after reperfusion

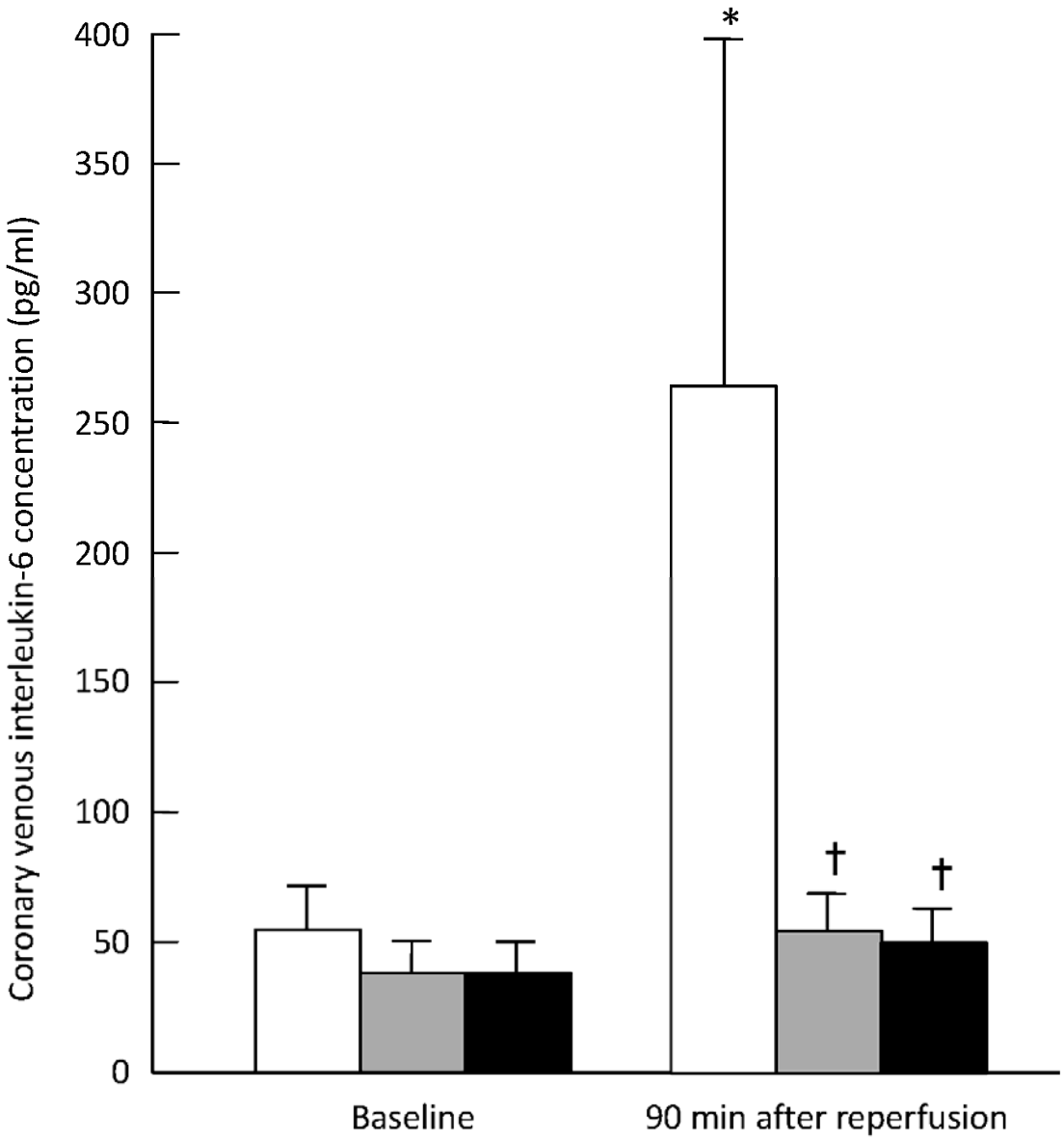

Figure 3. Coronary venous interleukin-6 concentrations of the LAD perfusion area before ischemia and 90 min after reperfusion. Values are mean±SD. N=7 in each group. *: $p<0.05$ vs. baseline; †: $p<0.05$ vs. group C. White bar, group C; gray bar, group L; black bar, group H

Table 1. Arterial blood gases and glucose

		P_0	R_{30}	R_{90}
PO_2	Group C	171±28	161±22	159±17
(mmHg)	Group L	180±27	173±14	167±16
	Group H	184±24	173±26	167±31
PCO_2	Group C	37±2	37±1	36±1
(mmHg)	Group L	37±1	37±1	38±1
	Group H	38±1	37±1	37±1
Glucose	Group C	101±15	107±14	107±12
(mg/dl)	Group L	103±12	110±14	104±12
	Group H	104±15	103±13	103±12

Values are mean±SD

P_0, baseline; R30 and R90, 30 and 90 minutes after reperfusion

Table 2. Systemic and coronary hemodynamics

		P_0	R_0	R_5	R_{30}	R_{60}	R_{90}
HR	Group C	94±13	94±17	97±10	95±16	95±20	94±15
	Group L	79±10	85±9	85±10	83±10	83±10	83±11
	Group H	82±15	82±18	80±17	82±19	80±19	80±20
LVdP	Group C	122±25	119±26	114±27	114±31	114±31	116±30
	Group L	109±19	104±16	105±15	104±14	100±15	107±15
	Group H	112±24	101±17	106±20	108±24	114±27	113±26
LVdP/dt_{max}	Group C	2881±531	2404±488	2314±540	2392±524	2306±524	2425±465
	Group L	2229±549	1966±524	2049±522	2030±441	2002±303	2109±471
	Group H	2413±353	2024±257	2049±396	2186±373	2252±295	2295±265
LVEDP	Group C	8.4±3.0	10.0±2.5	8.1±1.9	9.0±2.4	8.5±1.9	8.3±1.9
	Group L	9.2±2.8	10.3±2.8	10.1±2.8	9.0±2.3	8.7±1.9	8.9±2.2
	Group H	8.5±1.4	9.8±2.4	9.3±2.1	8.1±1.4	8.3±1.4	8.0±1.4
CPP	Group C	118±19	115±23	107±25	107±27	107±28	108±28
	Group L	110±19	107±18	102±15	104±15	100±16	105±16
	Group H	108±19	100±15	99±17	104±19	109±19	108±21
CBF	Group C	23.3±9.8	0*	44.8±8.2*	21.4±9.2	21.4±9.9	21.9±9.8
	Group L	25.4±8.3	0*	53.9±14.3*	21.1±7.6	22.9±7.0	24.2±8.4
	Group H	23.8±9.8	0*	47.0±13.9*	20.4±8.9	21.4±8.1	22.6±8.5
%SS	Group C	22.9±5.9	-3.4±3.7*	7.0±3.3*	7.5±3.8*	6.6±2.2*	7.2±3.2*
	Group L	21.5±4.6	-1.6±1.2*	8.5±2.2*	11.6±3.1*	13.7±3.3*†	15.1±3.3*†
	Group H	21.4±4.4	-1.7±1.6*	9.6±3.6*	12.8±4.4*	15.1±4.3*†	16.6±4.1*†

*: $p<0.05$ vs. P_0; †: $p<0.05$ vs. group C

Values are mean±SD

P_0, baseline; R0, just before reperfusion; R5, R30, R60 and R90, 5, 30, 60 and 90 minutes after reperfusion; HR, heart rate (beat/min); LVdP, left ventricular developed pressure (mmHg); LVdP/dtmax, maximal rate of increase of left ventricular developed pressure (mmHg/sec); LVEDP, left ventricular end-diastoric pressure (mmHg); CPP, coronary perfusion pressure (mmHg); CBF, coronary blood flow (ml/min); %SS, percentage of segment shortening