$$Vs = V_{RV} + V_{LA} + V_{RA} + V_{Ca,s} + V_{Cv,s} + V_{Ca,p,l} + V_{Cv,p,l} + V_{Ca,p,r} + V_{Cv,p,r} \quad (8)$$

We solved the simultaneous differential equations (Eqs. 1–8) using MATLAB/Simulink (MathWorks, Inc., MA, USA).

Shunt diameter was decreased stepwise from 4.0 to 3.0 mm in decrements of 0.5 mm in the original Norwood procedure model using a SPA shunt, and increased from 4.0 mm to 6.0 mm at increments of 1.0 mm in the modified Norwood procedure model using a RV-PA shunt. Diameter of the PA banding was increased from 1.5 to 2.0 mm in increments of 0.25 mm in the hybrid procedure model. Systemic and pulmonary flow (Q_s and Q_p), systemic and pulmonary arterial pressures (SAP and PAP), systemic-to-pulmonary diastolic run-off or diastolic regurgitation from PA to RV, right ventricular end-diastolic volume (RVEDV), stroke work (SW), systolic pressure–volume area (PVA), and mechanical efficiency (SW/PVA) after each procedure were calculated for each shunt or banding diameter. Heart rate and SAP widely vary in clinical settings. This makes it difficult to compare the procedural effects. Therefore, to provide a proper comparison among the three different procedures, heart rate was fixed at 150 bpm and mean SAP was set at the same value as that of the control state, by adjusting the total stressed blood volume (V_s).

Calculation of arterial and venous oxygen saturation

Arterial (SaO_2) and venous O_2 saturation (SvO_2) are calculated by the following equations for Q_p and Q_s (l/min):

$$SaO_2 = S_{PV}O_2 - \frac{CVO_2 \times BSA}{1.34 \times Hb \times 10 \times Q_p}$$

$$SvO_2 = SaO_2 - \frac{CVO_2 \times BSA}{1.34 \times Hb \times 10 \times Q_s}$$

where $S_{PV}O_2$ is the pulmonary venous O_2 saturation (0.97), CVO_2 is the whole body O_2 consumption (185 ml O_2/min/m^2), BSA is the body surface area (0.20 m^2), Hb is the hemoglobin concentration (16.0 g/dl), and 10 (dl/l) and 1.34 (ml O_2/g) are conversion factors [11, 16, 17].

Results

By adjusting the stressed blood volume, mean SAP and Q_s were maintained at around 58.7 mmHg and 0.83 l/min, respectively, in all the models.

In the hybrid procedure model, looser PA banding increased systolic SAP and decreased diastolic SAP. In the SPA shunt Norwood procedure model, systolic SAP increased and diastolic SAP decreased with the use of a larger shunt. In the RV-PA shunt Norwood procedure model, however, change in shunt diameter affected systolic and diastolic SAP only slightly (Fig. 2a). In all the models, mean PAP (Fig. 2b), Q_p (Fig. 2c) and Q_p/Q_s (Fig. 2d) increased with increase in diameter. These parameters were almost equivalent among the 1.75-mm hybrid procedure (13.7 mmHg, 0.97 l/min and 1.18, respectively), 3.5-mm SPA shunt (13.9 mmHg, 1.04 l/min and 1.27), and 6.0-mm RV-PA shunt (11.6 mmHg, 0.86 l/min and 1.05) models. The SaO_2 and SvO_2 also were elevated with increase in diameter (Fig. 2e). The SaO_2 and SvO_2 were almost equivalent among the 1.75-mm hybrid procedure (79.2 and 58.1 %, respectively), 3.5-mm SPA shunt (80.4 and 59.4 %), and 6.0-mm RV-PA shunt (77.0 and 56.1 %) models. Use of a larger caliber conduit in the SPA shunt Norwood procedure and a looser band in the hybrid procedure caused a significant increase in diastolic run-off from systemic to pulmonary circulation. Use of a larger caliber conduit in the RV-PA shunt Norwood procedure also caused an increase in diastolic regurgitation from PA to RV, but this increase was smaller than those in diastolic run-off (Fig. 2f).

The pressure–volume loops of the 1.75-mm hybrid procedure as well as the 3.5-mm SPA shunt and 6.0-mm RV-PA shunt Norwood procedures are shown in Fig. 3. Although RVEDV did not differ among the 1.75-mm hybrid procedure (23.6 ml), 3.5-mm SPA shunt (24.1 ml), and 6.0-mm RV-PA shunt (23.0 ml) (Fig. 4a), SW was slightly smaller in the 6.0-mm RV-PA shunt (873 mmHg ml) than in the 1.75-mm hybrid procedure (929 mmHg ml) or the 3.5-mm SPA shunt (974 mmHg ml) (Fig. 4b). Furthermore, systolic PVA in the 6.0-mm RV-PA shunt (992 mmHg ml) was significantly smaller than that in the 1.75-mm hybrid procedure (1,184 mmHg ml) or the 3.5-mm SPA shunt (1,228 mmHg ml) (Fig. 4c). As a result, mechanical efficiency (SW/PVA) was higher in the 6.0-mm RV-PA shunt (88.0 %) than in the 1.75-mm hybrid procedure (78.5 %) or the 3.5-mm SPA shunt (79.3 %) (Fig. 4d). The relation between Q_p/Q_s and mechanical efficiency was almost superimposed for the hybrid procedure and the SPA shunt Norwood procedure, but efficiency was much better in the RV-PA shunt Norwood procedure at all Q_p/Q_s levels (Fig. 5).

Discussion

Advantages of hybrid procedure

The present computational study revealed that the hemodynamics provided by the hybrid procedure is comparable to that by the conventional Norwood procedure using a SPA shunt, but is less favorable than that by the modified

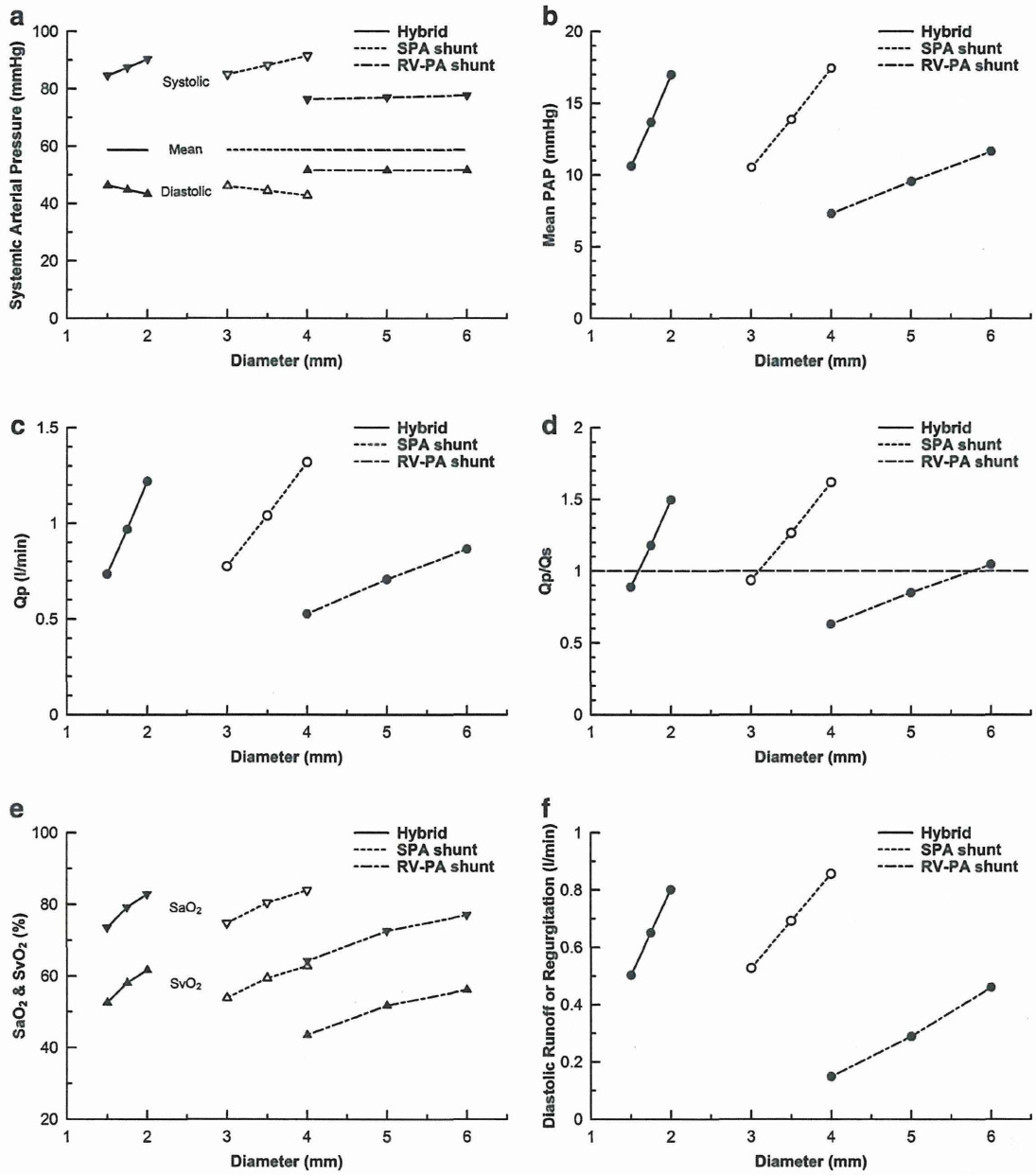

Fig. 2 The relations between shunt or banding diameter and systemic arterial pressure (**a**), mean pulmonary artery pressure (PAP, **b**), pulmonary flow (Q_p, **c**), ratio of pulmonary to systemic flow (Q_p/Q_s, **d**), arterial and venous saturation (SaO_2 and SvO_2, respectively, **e**) and diastolic run-off or regurgitation (**f**). Mean PAP was the average of the *left* and *right* mean PAP and Q_p was the sum of the *left* and *right* pulmonary flow in the hybrid procedure model. *Solid line*, hybrid procedure; *dotted line*, systemic to pulmonary artery (SPA) shunt; *dot-dashed line*, right ventricle to pulmonary artery (RV-PA) shunt

Norwood procedure using a RV-PA shunt. The 1.75-mm hybrid procedure was almost equivalent to the 3.5-mm SPA shunt Norwood procedure from the viewpoint of postoperative hemodynamics. Therefore, without using CPB, the hybrid procedure may be able to achieve similar clinical outcome as the SPA shunt Norwood procedure even in high-risk patients.

In low-weight infants, the Norwood procedure and its modification are a risk factor for increased mortality. Several studies have reported that infants weighing less than 2.5 kg have a significantly higher mortality after the Norwood procedure [18, 19]. The CPB procedure per se is unlikely to be a risk factor associated with early postoperative mortality due to recent improvements in cardiovascular

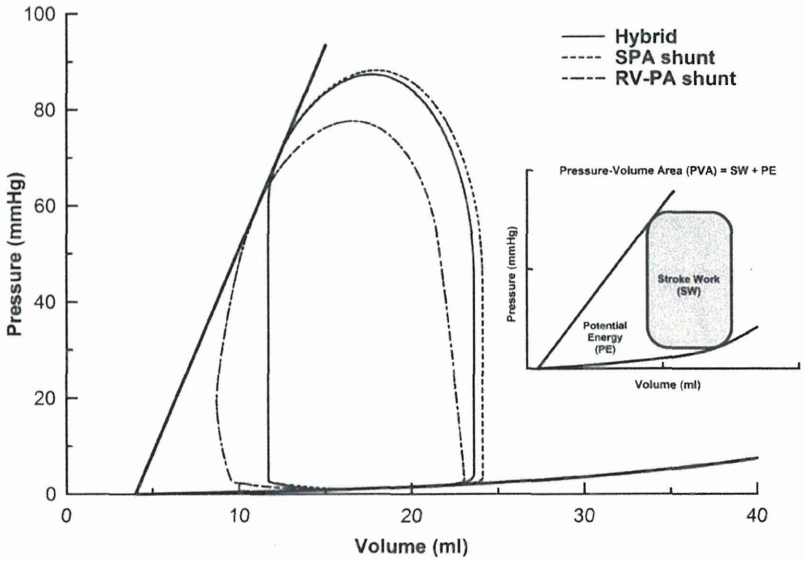

Fig. 3 Simulated pressure–volume loops of the 1.75-mm hybrid procedure (*solid line*), the Norwood procedure with a 3.5-mm systemic to pulmonary artery (SPA) shunt (*dotted line*) and Norwood procedure with a 6.0-mm right ventricle to pulmonary artery (RV-PA) shunt (*dot-dashed line*)

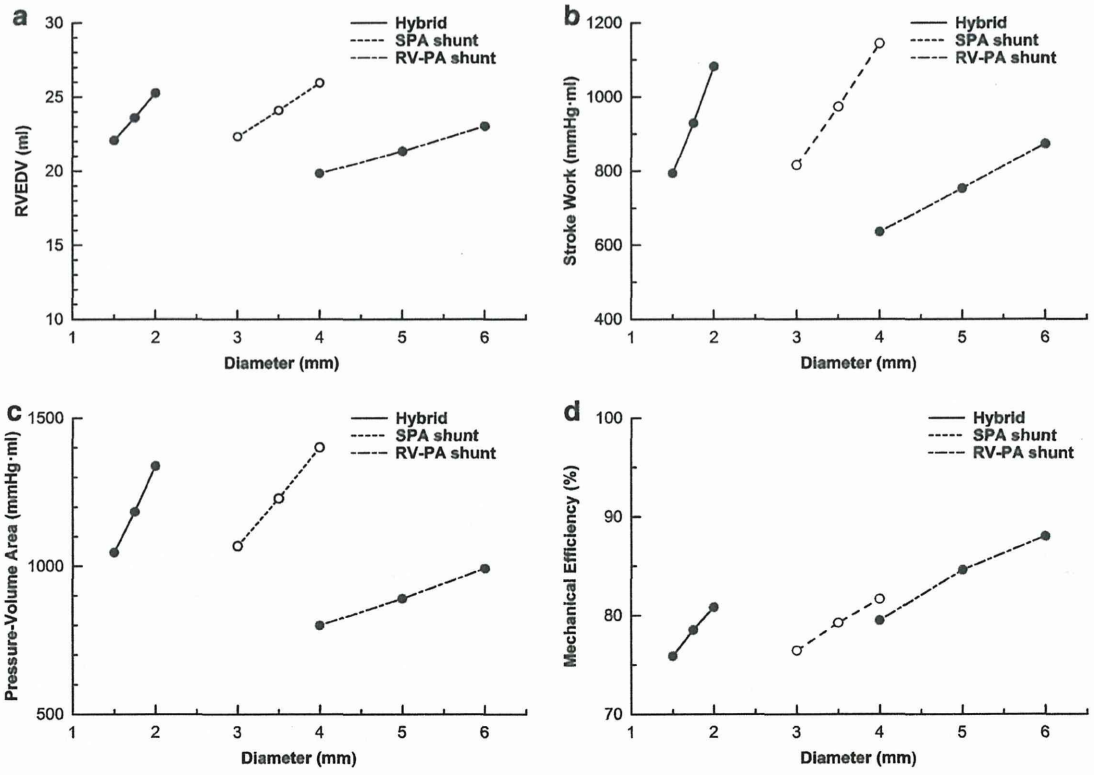

Fig. 4 The relations between shunt or banding diameter and right ventricular end-diastolic volume (RVEDV, **a**), stroke work (**b**), systolic pressure–volume area (**c**) and mechanical efficiency (**d**). *Solid line*, hybrid procedure; *dotted line*, systemic to pulmonary artery (SPA) shunt; *dot-dashed line*, right ventricle to pulmonary artery (RV-PA) shunt

management during CPB [20]. However, Pawade et al. [21] reported that the risk factors associated with an increased risk of early death in infants weighing less than 2.5 kg undergoing reconstruction surgery using CPB were (1) presence of preoperative metabolic acidosis; (2) univentricular repair; and (3) duration of cardiopulmonary bypass.

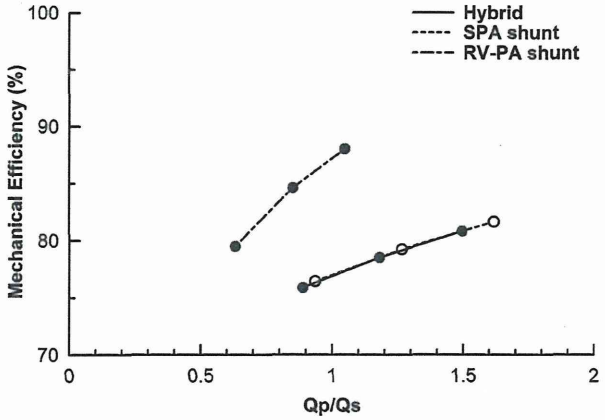

Fig. 5 The relation between Q_p/Q_s and mechanical efficiency. *Solid line*, hybrid procedure; *dotted line*, systemic to pulmonary artery (SPA) shunt; *dot-dashed line*, right ventricle to pulmonary artery (RV-PA) shunt

Weinstein et al. [22] also reported a trend toward increased mortality with increased CPB time in HLHS infants weighting less than 2.5 kg. Since the hybrid procedure does not require CPB, increased CPB time is not a concern. Pizarro et al. [23] have reported that patients undergoing hybrid palliation have lower preoperative blood pH than Norwood patients. This may reflect the surgeons' selection of hybrid procedure for high-risk infants. In their study, there were no significant differences in hospital and interstage mortalities between the hybrid and the conventional Norwood strategies. Because preoperative metabolic acidosis is associated with increased risk of early death after CPB [21], the most suitable patients for the hybrid procedure may be low-weight infants with preoperative metabolic acidosis.

Disadvantages of hybrid procedure

The hybrid procedure is very sensitive to a change in diameter of the banding site. A slight increase in diameter readily causes pulmonary over-circulation. In the present study, an increase in diameter of only 0.25 mm (from 1.75 to 2.0 mm) resulted in a 26.0 % increase in Q_p (Fig. 2c). Thus, surgeons have to adjust the band size carefully to avoid pulmonary over-circulation or malperfusion.

The present study provides important insight into ventricular energetics of the hybrid procedure. The hybrid procedure cannot reduce PVA while the Norwood procedure with RV-PA shunt can reduce PVA. The PVA in the 1.75-mm hybrid procedure was 19.3 % larger than that in the 6.0-mm RV-PA shunt Norwood procedure. Thus, the hybrid procedure maintains systemic circulation at the expense of higher oxygen consumption compared to the RV-PA shunt. Because coronary blood flow depends on myocardial oxygen demand under physiological conditions [24], the hybrid procedure requires more coronary blood flow than the RV-PA shunt to maintain the same systemic circulation. This feature may impair coronary flow reserve. The hybrid procedure may have poor tolerance to postoperative myocardial oxygen supply/demand imbalance.

The hybrid procedure as well as the SPA shunt Norwood procedure cannot avoid diastolic run-off from the systemic to pulmonary circulation. Therefore, the hybrid procedure requires more stressed blood volume to maintain systemic cardiac output (Q_s) at the same level as the Norwood procedure with the RV-PA shunt. This means that the hybrid procedure may easily cause volume overload to the single ventricle. In addition, the present study demonstrates that mechanical efficiency in the 1.75-mm hybrid procedure is 9.5 % lower compared to the 6.0-mm RV-PA shunt. Using the hybrid procedure, the RV must pump blood to a high-pressure system, i.e., the systemic circulation. Thereafter, a portion of the blood flows into a low pressure system, i.e., the pulmonary circulation, according to the pressure gradient. Using the RV-PA shunt, however, RV can simultaneously eject blood to both high- and low-pressure systems. This difference in mechanics results in increased elastic potential energy (Fig. 3) and reduced mechanical efficiency (SW/PVA) for the hybrid procedure compared to the RV-PA shunt. The impaired mechanical efficiency may be associated with the clinical observation that the hybrid procedure does not improve interstage mortality although this approach reduces the initial surgical insult [23].

Limitations

First, intraoperative use of cardiopulmonary bypass and circulatory arrest in the Norwood procedure may affect the postoperative ventricular performance. Furthermore, the right ventriculotomy in the Norwood procedure with the RV-PA shunt may impair ventricular contractility. These factors may reduce potential advantage on ventricular energetics of the Norwood procedure compared with the hybrid procedure. However, our previous simulation study has indicated the Norwood procedure with the RV-PA shunt still has an advantage on ventricular energetics even when end-systolic elastance is reduced by approximately 20 % [11].

Second, in the present study, mean arterial pressure and Q_s were fixed for all of the models, and the results need to be interpreted accordingly. In other words, actual ventricular performance could depend on a given hemodynamic state in each patient.

Third, a patient-specific simulation may be helpful to understand the patient's fluid dynamics [25]. However, our present model was based on the previous studies [11, 13–16] and not a patient-specific model. To perform a

patient-specific simulation, we have to develop an accurate method of estimating the parameters for simulation, such as elastance, resistance and compliance.

Conclusions

Although the hybrid procedure has the advantage of requiring no CPB, this procedure provides similar hemodynamics as the conventional Norwood procedure using a SPA shunt and has greater myocardial oxygen demand compared to the modified Norwood procedure using a RV-PA shunt.

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Conflict of interest The authors declare that they have no conflict of interest.

Ethical standards Because this study is a simulation study, the declaration about ethics does not apply to this study.

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Effects of intravenous cariporide on release of norepinephrine and myoglobin during myocardial ischemia/reperfusion in rabbits

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ABSTRACT

Aims: To examine the effects of cariporide, a Na^+/H^+ exchanger-1 inhibitor, on cardiac norepinephrine (NE) and myoglobin release during myocardial ischemia/reperfusion by applying a microdialysis technique to the rabbit heart.

Main methods: In anesthetized rabbits, two dialysis probes were implanted into the left ventricular myocardium and were perfused with Ringer's solution. Cariporide (0.3 mg/kg) was injected intravenously, followed by occlusion of the left circumflex coronary artery. During 30-min coronary occlusion followed by 30-min reperfusion, four consecutive 15-min dialysate samples (two during ischemia and two during reperfusion) were collected in vehicle and cariporide-treated groups. Dialysate myoglobin and NE concentrations were measured by immunochemistry and high-performance liquid chromatography, respectively.

Key findings: Dialysate myoglobin and NE concentrations increased significantly during myocardial ischemia/reperfusion in both vehicle and cariporide-treated groups ($P < 0.01$ vs. baseline). In cariporide-treated group, dialysate myoglobin concentrations were significantly lower than those in vehicle group throughout ischemia/reperfusion ($P < 0.01$ at 0–15 min of ischemia, $P < 0.05$ at 15–30 min of ischemia, $P < 0.01$ at 0–15 min of reperfusion, and $P < 0.01$ at 15–30 min of reperfusion). However, dialysate NE concentrations in cariporide-treated group were lower than those in vehicle group only during ischemia ($P < 0.01$ at 0–15 min of ischemia, and $P < 0.05$ at 15–30 min of ischemia).

Significance: When administered before ischemia, cariporide reduces myoglobin release during ischemia/reperfusion and decreases NE release during ischemia.

Introduction

The Na^+/H^+ exchanger isoform-1 (NHE-1) is a ubiquitously expressed integral membrane protein transporter that regulates intracellular pH by removing intracellular H^+ in exchange for extracellular Na^+ (Fliegel, 2005). NHE-1 has been reported to play an important role in the pathogenesis of myocardial ischemia/reperfusion injuries (Avkiran, 1999, 2003). During myocardial ischemia/reperfusion, the activity or quantity of NHE-1 increases, leading to an accumulation of intracellular Na^+, which in turn reduces and eventually reverses the driving force for the Na^+/Ca^{2+} exchanger, thereby decreasing Ca^{2+} efflux and eventually increasing Ca^{2+} influx. This process subsequently induces intracellular Ca^{2+} overload and promotes structural (apoptosis) and functional (arrhythmias, hypercontraction) damages (Leineweber et al., 2007). In sympathetic nerve endings, increased NHE-1 activity results in accumulation of axoplasmic Na^+ that diminishes the inward transport and eventually favors the outward transport of norepinephrine (NE) via the neuronal NE transporter (a bidirectional NE carrier, NET) (Leineweber et al., 2007). Thus, inhibition of NHE-1 may reduce NE release into the synaptic cleft. Because excessive NE release from sympathetic nerve endings is a prominent cause of arrhythmias and cardiac dysfunction (Leineweber et al., 2007), NHE-1 inhibitors may provide cardioprotection against functional damage during ischemia/reperfusion.

Cariporide, a NHE-1 inhibitor, has been reported to be a pharmacologically preconditioning agent. Several experimental studies have demonstrated that pretreatment with cariporide reduces infarct size (Kristo et al., 2004; Miura et al., 1997), suggesting that the inhibition of NHE-1 protects the heart from structural damage during ischemia/reperfusion. Furthermore, Létienne et al. (2006) reported that cariporide significantly reduced plasma myoglobin and troponin I

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0024-3205/

levels that strongly correlated with myocardial necrosis. Therefore, cariporide treatment before ischemia may reduce both pathological NE release and structural damage of the heart during ischemia/reperfusion. However, because of the limited methodology for simultaneous monitoring NE release and structural heart damage in the past, the mechanism of cardioprotection by cariporide has not been fully elucidated. Our group has already demonstrated that cardiac microdialysis technique can simultaneously monitor interstitial NE and myoglobin levels in the ischemic region of the left ventricle (Kitagawa et al., 2005). Because there is less blood flow in ischemic lesion, diffusion of myoglobin should be limited. Therefore interstitial myoglobin level monitored by cardiac microdialysis technique may serve as a more accurate index of structural damage of the heart than plasma myoglobin level. Using this technique, we investigated the effects of cariporide on both NE and myoglobin releases in the left ventricle during ischemia/reperfusion.

Materials and methods

Animal preparation

Animal care was provided in accordance with the *Guiding Principles for the Care and Use of Animals in the Field of Physiological Sciences* approved by the Physiological Society of Japan. All protocols were approved by the Animal Subject Committee of the National Cerebral and Cardiovascular Center. Twelve adult male Japanese white rabbits weighing from 2.5 to 3.5 kg were anesthetized with an intravenous injection of pentobarbital sodium (40 mg/kg) via the marginal ear vein, followed by continuous intravenous infusion of pentobarbital sodium (2 mg/kg/h). Butorphanol (0.1 mg/kg) was injected intramuscularly every 2–3 h for analgesia. Adequate anesthesia level was confirmed by loss of the ear pinch response. The rabbits were intubated and ventilated mechanically with room air mixed with oxygen. Systemic arterial pressure was monitored by a catheter inserted into the femoral artery. Heparin sodium (10 IU/kg/h) was infused to prevent blood coagulation in the femoral artery catheter. Heart rate was monitored on body surface electrocardiogram. Arterial pressure and heart rate were recorded by a PowerLab Data Acquisition System (ADInstruments, Dunedin, New Zealand). Esophageal temperature was maintained between 38 and 39 °C using a heating pad.

With the animal in the lateral position, the fifth or sixth rib on the left side was partially removed and a small incision was made in the pericardium to expose the heart. A snare was placed around the main branch of the left circumflex coronary artery (LCX) to act as an occluder for later coronary occlusion. Two dialysis probes were implanted in the left ventricular wall corresponding to the region perfused by the LCX. To confirm that the dialysis probes were properly located inside the ischemic region, we examined the color and motion of the ventricular wall during a brief occlusion. To avoid a preconditioning effect, the duration of brief occlusion was limited to a few seconds.

At the end of each experiment, the LCX was again occluded. Evans blue (1%) was intravenously injected to confirm that the dialysis probes were properly implanted within the ischemic area. The rabbits were euthanized by injecting an overdose of pentobarbital sodium. At postmortem, the heart was excised from the euthanized rabbit and was transversely sliced into 3 or 4 pieces. The left ventricular cavity was macroscopically examined to confirm that the dialysis membranes were not exposed to the left ventricular cavity.

Dialysis technique

Materials for cardiac microdialysis probe have been described in detail previously (Akiyama et al., 1991; Kitagawa et al., 2005). The long transverse dialysis probes were custom made. For monitoring myocardial interstitial NE levels, a dialysis fiber (length 8 mm, o.d. 0.31 mm, i.d. 0.20 mm; PAN-1200 50,000 molecular weight cutoff; Asahi Chemical Japan) was glued at both ends to polyethylene tubes. This dialysis probe was perfused with Ringer's solution at a rate of 2 μl/min using a microinjection pump (Carnegie Medicine CMA/102, Sweden). Each dialysate sample was collected over 15 min (1 sampling volume = 30 μl) into a microtube containing 3 μl of 0.1 N HCl to prevent amine oxidation. Dialysate NE level was measured by high-performance liquid chromatography with electrochemical detection (ECD-300, Eicom, Japan) as described in the Analytical procedures section.

For monitoring myocardial interstitial myoglobin levels, another dialysis probe (length 8 mm, o.d. 0.215 mm, i.d. 0.175 mm, 300 Å pore size; Evaflux type 5A; Kuraray Medical, Japan) was used as described previously (Kitagawa et al., 2005). This dialysis probe was perfused with Ringer's solution at a rate of 5 μl/min. Dialysate sampling period was 15 min (1 sampling volume = 75 μl). Dialysate myoglobin concentration was measured by immunochemistry using a Cardiac Reader (Roche Diagnostics, Basel, Switzerland) as described in the Analytical procedures section.

Experimental protocols were started 2 h after implantation of the dialysis probes. During dialysate sampling, we took into account the dead space between the dialysis membrane and the sample tube.

Analytical procedures

Dialysate NE concentrations were measured by high-performance liquid chromatography with electrochemical detection. An alumina procedure was employed to remove the interfering compounds from the dialysate samples. The liquid chromatography system consisted of a pump (EP-300, Eicom) with a degasser (DG-300, Eicom), a separation column (Eicompak CA-5ODS, Eicom), and an electrochemical detector (ECD-300, Eicom). The temperature of the separation was maintained at 25 °C by a column oven (ATC-300, Eicom). The electrochemical detector was operated with a graphite electrode (WE-3G, Eicom) at +0.45 V vs. an Ag/AgCl reference electrode. Mobile phase consisted of 12% (v/v) methanol, 1-octanesulfonic acid sodium (600 mg/l) and 88% (v/v) 100-mM phosphate buffer adjusted to pH 5.68. The pump flow rate was 0.23 ml/min. Chromatograms were recorded and analyzed by a laboratory computer connected with an A–D converter (Power Chrom EPC-500, Eicom). NE concentrations were determined by measuring the peak areas. The absolute detection limit of NE was 0.1 pg per injection (signal-to-noise ratio = 3).

Dialysate myoglobin concentrations were measured by the Cardiac Reader system (Roche Diagnostics). Single-use immunochemical test strips were used in the Cardiac Reader system. When a sample was added to the test well, the sample migrated along the strip due to capillary action, and myoglobin combined with two specific monoclonal antibodies. The resulting sandwich complex was immobilized by streptavidin in a stripe along the read window, producing a reddish line with an intensity related to myoglobin concentration. The CCD (Charge Coupled Device) camera quantified the intensity of the signal line and control line on the immunochemical test strips via reflectance measurements. The reflectance measurements were then converted into myoglobin concentration using electronically stored lot-specific calibration curves. The measuring range was between 30 and 700 ng/ml (Ambrose et al. 2002). When dialysate myoglobin concentrations were expected to be higher than 700 ng/ml, dialysate samples were diluted 10 or 100 times with saline.

Experimental protocols

Time courses of dialysate NE and myoglobin concentrations during acute myocardial ischemia/reperfusion (n = 6, vehicle group)

We examined the time courses of dialysate NE and myoglobin concentrations during 30 min of ischemia followed by 30 min of reperfusion. After 15-min baseline sampling, the main branch of the LCX was occluded for 30 min and then was released. Four consecutive 15-min

dialysate samples were collected during coronary occlusion (30 min) and reperfusion (30 min).

Influence of cariporide on time courses of dialysate NE and myoglobin concentrations during acute myocardial ischemia/reperfusion (n = 6, cariporide-treated group)

We examined the effects of cariporide on NE and myoglobin releases during ischemia/reperfusion. Ten-mg powder of cariporide (Santa Cruz Biotechnology, Inc., CA, USA) was dissolved in 10-ml saline. After 15-min baseline sampling, cariporide of 0.3 mg/kg, which reportedly caused a sufficient reduction in infarct mass (Linz et al., 1998), was injected intravenously before coronary occlusion. The LCX occlusion and reperfusion were performed as described in the vehicle group, and four consecutive dialysate samples were collected.

Statistical methods

All data are presented as mean ± standard error. For each protocol, heart rate and mean arterial pressure were compared by one-way repeated measures analysis of variance followed by a Dunnett's test versus baseline. After logarithmic transformation, dialysate NE and myoglobin concentrations were compared by one-way repeated measures analysis of variance followed by a Dunnett's test versus baseline. The differences between two groups were compared by unpaired *t*-test. Statistical significance was defined as $P < 0.05$.

Results

Time courses of heart rate and mean arterial pressure

The time courses of heart rate and mean arterial pressure during myocardial ischemia/reperfusion are shown in Table 1. In the vehicle group, coronary occlusion did not affect heart rate or mean arterial pressure. After reperfusion, heart rate decreased significantly but slightly to 266 ± 6 bpm at 7.5 min (compared with baseline: 277 ± 5 bpm; $P < 0.05$) and 265 ± 4 bpm at 22.5 min of reperfusion ($P < 0.05$ vs. baseline). In the cariporide-treated group, heart rate and mean arterial pressure did not change throughout ischemia and reperfusion.

There were no significant differences between the vehicle and cariporide-treated groups in heart rate and mean arterial pressure throughout the experiment.

Time course of dialysate NE concentration

Time course of dialysate NE concentration is shown in Fig. 1. In some baseline samples, dialysate NE concentrations were below the detection limit (0.1 pg/30-μl injection). For statistical analysis, baseline values were represented by the detection limit of 3.7 pg/ml.

In the vehicle group, dialysate NE concentration increased to 7251 ± 1891 pg/ml at 0–15 min of ischemia ($P < 0.01$ vs. baseline), reaching a peak of 56,586 ± 10,972 pg/ml at 15–30 min of ischemia ($P < 0.01$ vs. baseline). After reperfusion, dialysate NE concentration decreased to 16,837 ± 3906 pg/ml at 0–15 min ($P < 0.01$ vs. baseline), and further to 675 ± 243 pg/ml at 15–30 min of reperfusion ($P < 0.01$ vs. baseline).

In the cariporide-treated group, dialysate NE concentration increased significantly to 1174 ± 273 pg/ml at 0–15 min of ischemia ($P < 0.01$ vs. baseline), reaching a peak of 29,278 ± 4138 pg/ml at 15–30 of ischemia ($P < 0.01$ vs. baseline). After reperfusion, dialysate NE concentration decreased to 11,913 ± 3145 pg/ml at 0–15 min ($P < 0.01$ vs. baseline), and further to 414 ± 133 pg/ml at 15–30 min of reperfusion ($P < 0.01$ vs. baseline).

Dialysate NE concentrations in the cariporide-treated group were significantly lower than those in the vehicle group during ischemia ($P < 0.01$ at 0–15 min and $P < 0.05$ at 15–30 min). However, there were no significant differences in dialysate NE concentration between two groups during reperfusion.

Table 1
Heart rate and mean arterial pressure during acute myocardial ischemia/reperfusion.

	Baseline	Ischemia 7.5 min	Ischemia 22.5 min	Reperfusion 7.5 min	Reperfusion 22.5 min
Vehicle (n = 6)					
Heart rate (bpm)	277 ± 5	269 ± 6	267 ± 4	266 ± 6*	265 ± 4*
Mean arterial pressure (mm Hg)	84 ± 4	80 ± 5	82 ± 5	82 ± 6	82 ± 6
Cariporide (n = 6)					
Heart rate (bpm)	274 ± 4	278 ± 6	276 ± 5	271 ± 6	269 ± 3
Mean arterial pressure (mm Hg)	80 ± 2	85 ± 3	85 ± 3	78 ± 2	75 ± 1

Data are expressed as mean ± standard error. *P < 0.05 by ANOVA followed by Dunnett's test versus baseline.

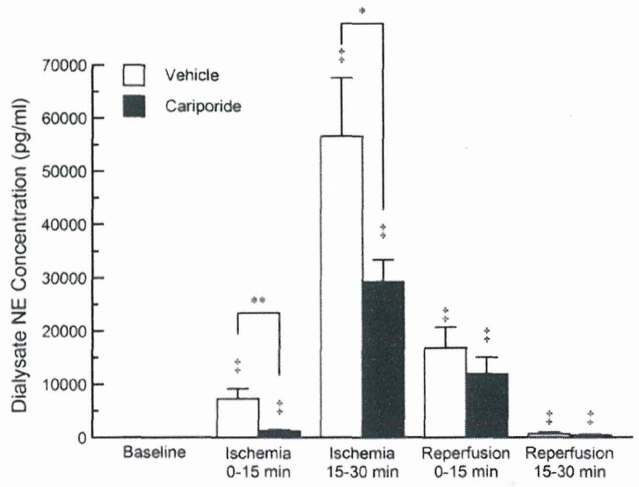

Fig. 1. Time courses of dialysate norepinephrine (NE) concentration during 30 min of ischemia followed by 30 min of reperfusion. Each dialysate sample was collected over a period of 15 min. Data are expressed as mean ± standard error. ‡P < 0.01, by ANOVA followed by Dunnett's test versus baseline; *P < 0.05 and **P < 0.01, by unpaired *t*-test.

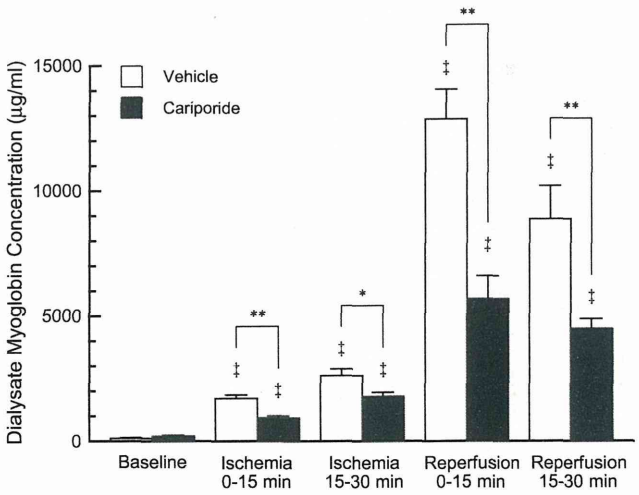

Fig. 2. Time courses of dialysate myoglobin concentration during 30 min of ischemia followed by 30 min of reperfusion. Each dialysate sample was collected over a period of 15 min. Data are expressed as mean ± standard error. ‡P < 0.01, ANOVA followed by Dunnett's test versus baseline; *P < 0.05 and **P < 0.01, by unpaired t test.

Time course of dialysate myoglobin concentration

Time course of dialysate myoglobin concentration is shown in Fig. 2. In the vehicle group, dialysate myoglobin concentration increased significantly from 128 ± 25 ng/ml at baseline to 1717 ± 137 ng/ml at 0–15 min of ischemia ($P < 0.01$ vs. baseline), and further to 2630 ± 262 ng/ml at 15–30 min of ischemia ($P < 0.01$ vs. baseline). After reperfusion, dialysate myoglobin concentration reached a peak of 12,887 ± 1186 ng/ml at 0–15 min ($P < 0.01$ vs. baseline), followed by a gradual decline (8903 ± 1317 ng/ml at 15–30 of after reperfusion, $P < 0.01$ vs. baseline).

In the cariporide-treated group, dialysate myoglobin concentration increased significantly from 218 ± 38 ng/ml at baseline to 943 ± 80 ng/ml at 0–15 min of ischemia ($P < 0.01$ vs. baseline), and further to 1798 ± 169 ng/ml at 15–30 min of ischemia ($P < 0.01$ vs. baseline). After reperfusion, dialysate myoglobin concentration reached a peak of 5690 ± 924 ng/ml at 0–15 min ($P < 0.01$ vs. baseline), followed by a gradual decline (4500 ± 395 ng/ml at 15–30 min of reperfusion, $P < 0.01$ vs. baseline).

Dialysate myoglobin concentrations in the cariporide-treated group were significantly lower than those in the vehicle group throughout ischemia/reperfusion ($P < 0.01$ at 0–15 min of ischemia, $P < 0.05$ at 15–30 min of ischemia, $P < 0.01$ at 0–15 min of reperfusion and $P < 0.01$ at 15–30 min of reperfusion).

Discussion

The present study demonstrated that intravenous injection of cariporide before coronary occlusion significantly reduced interstitial myoglobin levels during ischemia/reperfusion, and suppressed NE release from sympathetic nerve endings during ischemia but not during reperfusion.

NHE-1 inhibition and NE release

During acute myocardial ischemia, excessive NE release from sympathetic nerve endings and reduced NE reuptake into nerve endings may cause functional damages such as life-threatening arrhythmia. There are two major processes of NE release from sympathetic nerve endings. Under physiological conditions, NE is mainly released via Ca^{2+}-dependent exocytosis. In myocardial ischemia, however, the predominant process of NE release is Ca^{2+}-independent nonexocytosis via NET (Kurz et al., 1995). Physiologically, NET relocates NE within the synaptic cleft into the axoplasm, where NE is taken up into storage vesicles or degraded by monoamine oxidase. The NE vesicular storage depends on the pH gradient across the vesicular membrane maintained by an ATP-dependent H^+ pump. Increase in H^+ due to lowered pH as well as ATP depletion during ischemia leads to an increase in free axoplasmic NE (Leineweber et al., 2007), and activates the influx of Na^+ via NHE-1. Since the direction of NET-mediated transport depends on the Na^+ gradient across the membrane of sympathetic nerve terminals (Schömig et al., 1991), a rise in axoplasmic Na^+ concentration during ischemia diminishes the inward transport and favors the outward transport of NE, causing excessive Ca^{2+}-independent nonexocytotic NE release (Leineweber et al., 2007). Thus, by inhibiting NHE-1, cariporide may reduce the influx of Na^+ and suppress nonexocytotic NE release during ischemia. The present study proved that cariporide significantly reduces interstitial NE levels during ischemia. Therefore, cariporide may suppress functional damage caused by excessive NE release during ischemia.

This study also provided important evidence that cariporide does not reduce NE release during reperfusion. Thus, the effects of cariporide against excessive NE release may be limited to the ischemic period but not during reperfusion. This may be a reason why several clinical trials failed to prove the cardioprotective effects of NHE-1 inhibitors administered shortly before reperfusion. In the ESCAMI (Evaluation of the Safety and Cardioprotective Effects of Eniporide in Acute Myocardial Infarction) trial, administration of eniporide before reperfusion in patients with acute myocardial infarction did not improve clinical outcomes (death, cardiogenic shock, heart failure, life-threatening arrhythmias) (Zeymer et al., 2001). A previous study demonstrated that myocardial interstitial NE level decreased while dihydroxyphenylglycol (a metabolite of NE) level increased rapidly after reperfusion (Akiyama and Yamazaki, 2001). Thus, metabolites of catecholamine may also be associated with functional damage during reperfusion. Further investigations are necessary to clarify the effects of NHE-1 inhibitors on functional damage during reperfusion.

NHE-1 inhibition and myoglobin release

During myocardial ischemia, anaerobic glycolysis and ATP degradation produce H^+ that activates the influx of Na^+ via NHE-1. However, Na^+ efflux is attenuated because the Na^+/K^+-ATPase is inhibited during ischemia. Therefore, the net result enhanced Na^+ influx and reduced Na^+ efflux. An accumulation of intracellular Na^+ induces cytoplasmic Ca^{2+} overload via reverse-mode Na^+/Ca^{2+} exchanger, resulting in structural damage during myocardial ischemia/reperfusion (Leineweber et al., 2007). Therefore, cariporide may reduce Na^+ influx and suppress Ca^{2+} overload, resulting in the reduction of structural damage indicated by myoglobin release. Furthermore, several possible mechanisms of cardioprotective effects of cariporide have already been suggested. Nuñez et al. (2011) reported that attenuation of calcium-induced permeability transition pore opening after protein kinase C (PKC)-mediated mitochondrial ATP-sensitive potassium channel activation was a crucial step for the cardioprotective effects of cariporide. On the other hand, Ajiro et al. (2011) reported that platelet-activating factor (PAF) stimulated cardiac NHE-1 via the PAF receptor and signal relay required participation of the mitogen-activated protein kinase cascade. They also reported that PKC might not be involved in the stimulation of NHE-1 because PKC inhibitors did not significantly reduce the responses to PAF. Further investigations are clearly needed to identify the mechanisms.

Létienne et al. (2006) reported that cariporide significantly reduced plasma myoglobin level that strongly correlated with myocardial necrosis. However, we have previously demonstrated that plasma myoglobin level responds less sensitively than myocardial interstitial myoglobin level monitored by cardiac microdialysis (Kitagawa et al., 2005). Although a significant change in plasma myoglobin level occurs at 45–60 min after coronary occlusion in a rabbit ischemia model (Kitagawa et al., 2005), the myocardial microdialysis technique can detect a significant change in interstitial myoglobin level within 15 min after occlusion. The present study demonstrated that cariporide reduced interstitial myoglobin level from the early phase (0–15 min) of myocardial ischemia and this effect was sustained even after reperfusion was started. Several experimental studies have reported that preconditioning with cariporide salvages myocytes and reduces the release of cardiac-specific enzymes (Cun et al., 2007; Haist et al., 2003). Furthermore, the GUARDIAN (Guard During Ischemia Against Necrosis) trial revealed a significant correlation between elevated creatine kinase myocardial band (CK-MB) during the initial 48 h after coronary artery bypass grafting (CABG) and significantly increased six-month mortality (Gavard et al., 2003). A subgroup analysis of the GUARDIAN data revealed that a 120-mg dose of cariporide significantly reduced the combined incidence of death and myocardial infarction in patients undergoing high-risk CABG surgery, and that this benefit was sustained for 6 months (Boyce et al., 2003). Therefore, a reduction in the release of cardiac enzymes after reperfusion may have a close relation to the improvement of surgical outcomes. In the present study, cariporide also suppressed peak interstitial myoglobin level after reperfusion. Thus, cariporide administration before ischemia may be an effective cardioprotective strategy against structural damage during ischemia/reperfusion.

On the other hand, several studies have demonstrated that treatment with cariporide shortly before reperfusion does not