Original Research Article

Effects of intravenous cariporide on norepinephrine and myoglobin releases 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 releases 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 $P < 0.05$ 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.

Keywords: cariporide; myoglobin; norepinephrine; ischemia/reperfusion.

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. 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 ischemic region of the left ventricle (Kitagawa et al. 2005). Interstitial myoglobin level may serve an index of structural damage of the heart. 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 hours 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. 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 rabbits were euthanized by injecting an overdose of pentobarbital sodium. At postmortem, the left ventricular cavity was examined to confirm that the dialysis probes were implanted within the left ventricular myocardium.

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 previously (Akiyama et al. 1991).

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, Swiss).

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

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 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. After 15-min baseline sampling, cariporide (0.3 mg/kg) 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 (0.7 pg/injection). For statistical analysis, baseline values were represented by the detection limit of 23 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 56586 ± 10972 pg/ml at 15-30 min

of ischemia ($P < 0.01$ vs. baseline). After reperfusion, dialysate NE concentration decreased to 16837 ± 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 29278 ± 4138 pg/ml at 15-30 of ischemia ($P < 0.01$ vs. baseline). After reperfusion, dialysate NE concentration decreased to 11913 ± 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.

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 12887 ± 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 2045 ± 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 $P < 0.05$ 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 lead 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. Several clinical trials failed to prove the cardioprotective effects of NHE-1 inhibitors administered shortly before reperfusion. In the ESCAMI 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 is 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 reduction of structural damage indicated by myoglobin release.

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 releases 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 hours 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 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 reduce infarct size (Miura et al. 1997; Reffelmann and Kloner 2003). Klein et al. (2000) reported that reduction of infarct size was detectable only when cariporide was infused during the first 30 min after ischemia. In their study, cariporide infused after 45 min of ischemia until 10 min of reperfusion failed to reduce infarct size. In the ESCAMI (Evaluation of the Safety and Cardioprotective Effects of Eniporide in Acute Myocardial Infarction) trial, pretreatment with eniporide in patients undergoing reperfusion therapy for acute ST-elevation myocardial infarction did not reduce infarct size assessed by cardiac enzyme release (Zeymer et al. 2001). Thus, the reduction of interstitial myoglobin level during reperfusion observed in the present study may reflect amelioration of structural damage caused by ischemia and not necessarily the damage induced by reperfusion. Nevertheless, cariporide has certain cardioprotective effect against structural damage during ischemia and reperfusion.

Conclusions

Intravenous cariporide significantly reduced myocardial interstitial myoglobin level during ischemia/reperfusion and decreased NE release during ischemia. When administered before ischemia, treatment with cariporide may be an effective cardioprotective strategy against structural damage during ischemia/reperfusion and excessive NE release during ischemia.

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Figure legends

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.

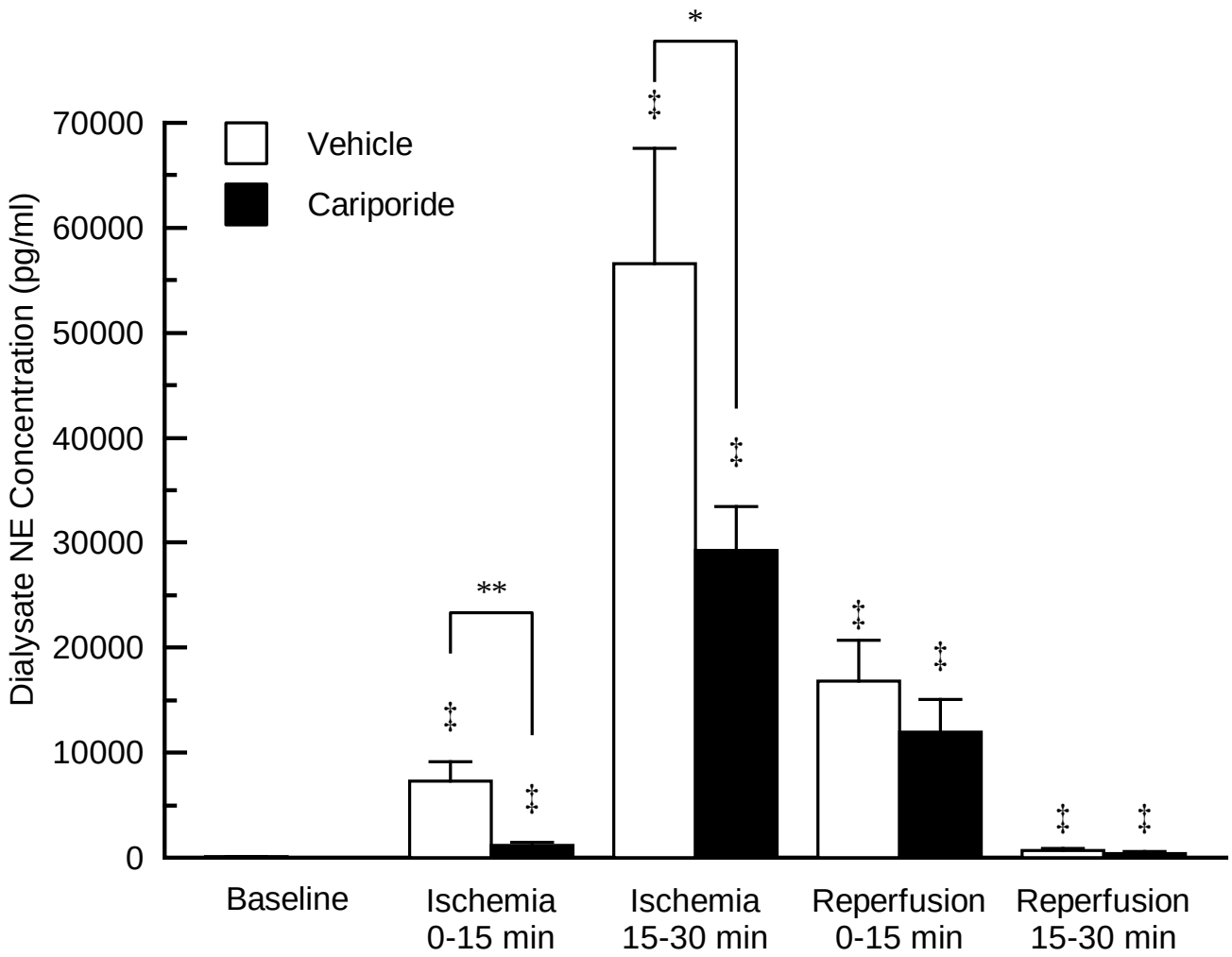
Fig. 2. Time courses of dialysate myoglobin concentration during 30 min of ischemia followed by 30 min of reperfusion. Each dialysate sample was collected over a period of 15 min. Data are expressed as mean ± standard error. ‡P < 0.01, ANOVA followed by Dunnett's test versus baseline; *P < 0.05 and **P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

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 (mmHg)	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 (mmHg)	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


Vehicle
Cariporide
Dialysate NE Concentration (pg/ml)
70000
60000
50000
40000
30000
20000
10000
0
*
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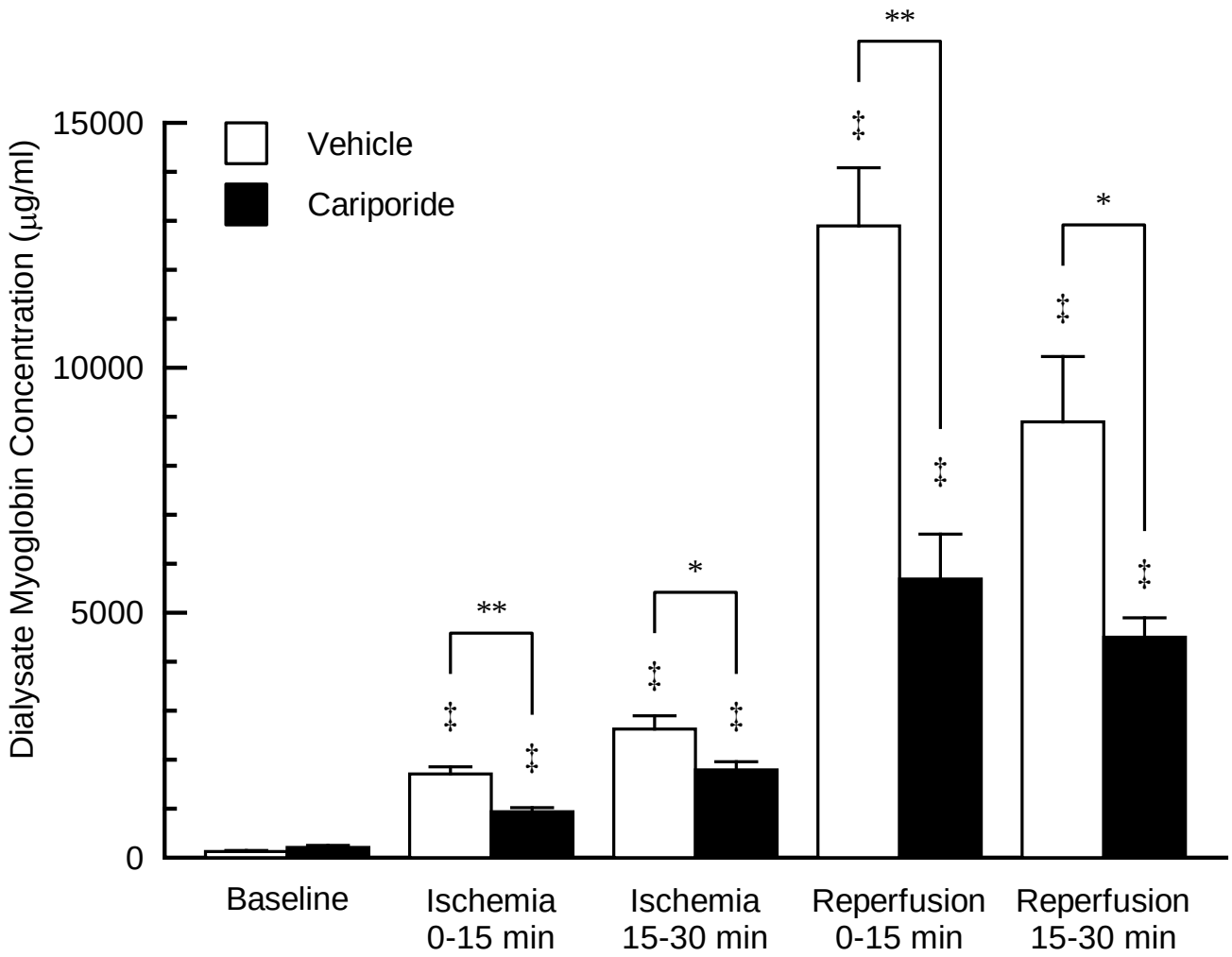
Baseline
Ischemia 0-15 min
Ischemia 15-30 min
Reperfusion 0-15 min
Reperfusion 15-30 min

Fig. 2


Dialysate Myoglobin Concentration (μg/ml)
15000
10000
5000
0
Vehicle
Cariporide
**
*
**
*
‡
Baseline
Ischemia 0-15 min
Ischemia 15-30 min
Reperfusion 0-15 min
Reperfusion 15-30 min