RSR13, a Synthetic Allosteric Modifier of Hemoglobin, Improves Myocardial Recovery Following Hypothermic Cardiopulmonary Bypass

Kenneth S. Kilgore, PhD; Charles F. Shwartz, MD; Marsha A. Gallagher, BS; Robert P. Steffen, PhD; Ralph S. Mosca, MD; Steven F. Bolling, MD

Background—During hypothermic blood cardioplegia, oxygen delivery to myocytes is minimal with ineffective anaerobic metabolism predominating. RSR13, 2-[4-[(3,5-dimethylanilino) carbonyl][methyl]phenoxy]-2-methylpropionic acid, a synthetic allosteric modifier of hemoglobin (Hb), increases release of oxygen from Hb, increasing oxygen availability to hypoxic tissues, and reverses the hypothermia-dependent increase in Hb oxygen affinity. We studied recovery of myocardial mechanical and metabolic function and examined myocardial morphology after cardioplegia, comparing RSR13 (1.75 mmol/L)-supplemented blood (RSR13-BC) to standard blood cardioplegia (BC).

Methods and Results—Twelve dogs underwent 15 minutes of 37°C global ischemia on cardiopulmonary bypass, followed by 75 minutes of hypothermic cardioplegia (13°C) with either BC (n=6) or RSR13-BC (n=6). There were no differences in baseline function between groups. Cardiac function was assessed after 30 minutes of 37°C reperfusion (BC versus RSR13-BC, respectively) by measuring: % return to normal sinus rhythm (0/100%), % of baseline dP/dt (33.7±1.7/76.3±1.9), % of baseline −dP/dt (26.6±2.0/81.1±1.6), stroke volume (3.5±0.5/7.1±0.9 mL), cardiac output (340±20/880±40.3 mL/min), and LVEDP (11.3±2.2/0.3±2.9 mm Hg). Postischemic oxidative and metabolic parameters including myocardial lactate, pyruvate, ATP content, and percent water content also were determined. Histological analysis demonstrated preservation of endothelial and myocyte morphology in hearts receiving RSR13-BC compared with BC.

Conclusions—These results indicate that in the setting of hypothermic cardiopulmonary bypass, RSR13 improves recovery of myocardial mechanical and metabolic function compared with standard hypothermic BC. Findings from this study suggest that RSR13-BC, by decreasing hemoglobin oxygen affinity, improves oxidative metabolism and preserves cellular morphology, resulting in significantly improved contractile recovery on reperfusion. (Circulation. 1999;100[Suppl II]:II-351–II-356.)

Key Words: cardiopulmonary bypass ■ hemoglobin ■ surgery

RSR13, 2-[4-[(3,5-dimethylanilino carbonyl)[methyl]phenoxy]-2-methylpropionic acid (Figure 1), is a synthetic allosteric modifier of hemoglobin (Hb) that acts to increase release of oxygen from Hb to the surrounding tissues.1-3 RSR13 acts to decrease Hb oxygen affinity through stabilization of deoxyHb in a manner similar to the natural allosteric effector of Hb, 2,3-diphosphoglycerate.4,5 Of additional importance is the ability of RSR13 to reverse the hypothermia-dependent increase in Hb oxygen affinity.6 An end result of RSR13 administration in vivo is a right shift in the hemoglobin/oxygen affinity curve and increase oxygen availability to hypoxic tissue.7

Previous studies have assessed RSR13 in a wide array of clinically relevant experimental models. Potential applications for the use of RSR13 include acting in the capacity of a radiosensitizer by increasing the response of tumor cells to radiation.3,8 Wei et al9 noted that RSR13 reversed the cerebral vasodilation associated with hypoxia and hypotension in the feline. In addition, RSR13 has been demonstrated to increase oxygen delivery to the brain and subsequently decrease cerebral infarct size after occlusion of the middle cerebral artery without affecting hemodynamics.10 Pagel et al11 examined the ability of RSR13 to preserve myocardial function after episodes of ischemia and reperfusion in the anesthetized canine. It was found that RSR13 significantly improved the functional recovery of stunned myocardium without affecting systemic or coronary hemodynamics, suggesting that increased oxygen availability by decreasing Hb oxygen binding affinity may be of benefit in the setting of myocardial ischemia.11 Although RSR13 has been demonstrated to be beneficial in the stunned myocardium, the cardioprotective effects of RSR13 in the setting of surgically-induced myocardial ischemia, such as that noted in cardiopulmonary bypass, have yet to be determined.
Clinical cardiac surgery has become safe and effective with current myocardial protection techniques using an assortment of cardioplegia solutions and variations in cardiac temperature.12,13 However, extended operative times associated with the advent of more complex surgical procedures have increased the demands on the approaches designed to promote increased myocardial protection. Of importance in surgically induced myocardial ischemia is the depletion of energy status from anaerobic metabolism. When reperfusion occurs, depletion of these energy stores is slow, and posts ischemic return of myocardial function is poor.14,15 Thus, agents designed to promote oxygen dissociation from hemoglobin to improve oxygen availability to ischemic tissue may be beneficial in surgical procedures that induce extended periods of myocardial ischemia, particularly in high-risk patients.

Recent reports have indicated that RSR13 preserves cardiomyocyte intracellular pH and high-energy phosphate content during myocardial ischemia, likely by improving oxygen delivery to ischemic tissue independent of changes in coronary or systemic hemodynamics.16 Maintenance of even minimal aerobic metabolism may achieve sufficient myocardial protection, perhaps owing to better generation and/or preservation of energetics, to augment ischemic tolerance.16–18 The objective of our study was to determine functional recovery and tissue preservation in the setting of cardiopulmonary bypass by the synthetic allosteric modifier of hemoglobin, RSR13. Myocardial metabolic and hemodynamic parameters, including load independent indices of cardiac function, were compared before and after bypass in a canine model of cardiopulmonary bypass that closely simulates the conditions of clinical hypothermic-cardiac surgery.

Methods

Guidelines for Animal Research

The procedures used in this study were in agreement with the guidelines of the Internal Review Board of the University of Michigan and approved by The University of Michigan Committee on the Use and Care of Animals. Veterinary care was provided by the University of Michigan Unit for Laboratory Animal Medicine. The University of Michigan is accredited by the American Association for Accreditation of Laboratory Animal Care, and the animal care use program conforms to the standards in The Guide for the Care and Use of Laboratory Animals (NIH publication 86-23).

Surgical Preparation

The surgical procedures used in this study have been described previously.19 Briefly, 12 adult mongrel dogs of either sex, weighing 22 to 28 kg, were studied in a surgical model of acute global myocardial normothermic ischemia followed by hypothermic cardioplegic protection and normothermic reperfusion. The animals were anesthetized with intravenous pentobarbital sodium (30 mg/kg), and anesthesia was maintained with intermittent dosing of pentobarbital sodium as needed. Endotracheal intubation was performed, and the animals were placed on a volume cycle ventilator with a tidal volume of 15 mL/kg and a FiO2 of 1.0. Aortic pressure was measured with a fluid-filled catheter placed in the intrathoracic aorta at the level of the left atrium. A pulmonary artery catheter was placed and the aortic and pulmonary artery catheters connected to a pressure monitor and recorded (Hewlett Packard, 78204 A). A median sternotomy was performed, the azygous vein was ligated, and the aortopulmonary space was dissected for placement of ascending aorta and main pulmonary artery transit-time ultrasonic flow probes (Transonic Systems Inc). Hemispheric piezoelectric, sonomicrometric crystals (3 MHz, 2.5 mm diameter, Channel Industries) were sutured to the epicardium of the left ventricle in the major and minor axis orientation. A third pair of flat crystals measured left ventricular free wall thickness. Left ventricular major axis, minor axis, and wall thickness dimensions were measured continuously by sonomicrometry (Triton Technology). A high-fidelity pressure transducer catheter (Millar Instruments) was placed through a puncture site in the apex of the left ventricle for measuring left intraventricular pressure. Cardiopulmonary bypass was instituted through a 2-stage venous cannula (DLP Inc) positioned in the right atrium and arterial cannula placed in the femoral artery. Systemic temperature was maintained at 34°C to 36°C during cardiopulmonary bypass. Thermistor probes were placed in the distribution of the left anterior descending and circumflex arteries to continuously monitor intramyocardial temperature (Yellow Springs Instrument Co). Arterial and cardiopulmonary temperatures were monitored by in-line thermistor probes (12100, Sarns Inc).

Investigational Compound

The sodium salt of RSR13, 2-[4-[(3,5-dimethylanilino carbonyl)methyl]phenoxy]-2-methylpropionic acid, was provided by Allos Therapeutics (Denver, Colo). RSR13 was dissolved in sterile 0.45% sodium chloride solution immediately before use and syringe filtered using a 0.2-μm filter. The solution was placed into 1 L standard blood cardioplegia (BC) to achieve a final concentration of 1.75 mmol/L. Total volume of RSR13 added was 10 mL. Control hearts received vehicle (0.45% sodium chloride-supplemented BC only. The final volume of cardioplegia was 1 L and was used in a recirculating fashion.

Experimental Protocol

The experimental protocol is outlined in Figure 2 and consisted of 15 minutes of normothermic global ischemia (no coronary flow) obtained by cross-clamping the aorta and opening the aortic root cannula. After the 15 minutes of normothermic ischemic insult, myocardial arrest was induced with either standard antegrade BC or BC supplemented with RSR13 (RSR13-BC; 1.75 mmol/L final concentration). Standard BC was used as the basis for both experimental groups throughout the experiments and consisted of blood mixed 4:1 with either high potassium solution (used for initial arrest) or low potassium solution (used for subsequent dosing). The cardioplegia had a hematocrit of 8% to 10% and was 100% saturated with oxygen. Both groups received initial arresting cardioplegia (30 mL/kg) at 10°C through the aortic root cannula and then repeat antegrade perfusion (15 mL/kg) every 15 minutes. Saline slush was placed around the heart in both groups to emulate clinical conditions and ensure complete cooling.

The hearts were maintained in cardioplegic arrest for 90 minutes; total ischemic time was 105 minutes. Following this, the aortic cross clamp was removed. After 30 minutes reperfusion, animals were able to be weaned from cardiopulmonary bypass. No inotropic agents
were administered. Hemodynamic data were collected using a Data Integrated Scientific Systems Recorder (Pinckney) and in-house programmed Augury Software package (Coyote Bay Instruments). Data were collected over a range of filling conditions of the left ventricle by slowly draining into the venous reservoir. Emptying curves were collected in triplicate before aortic cross clamping and after weaning from bypass. Animals were euthanized at the end of the protocol by infusion of a saturated KCl solution.

Sonomicrometric data were analyzed to determine the ventricular volume based on a prolate ellipsoid model of the left ventricle using the equation: \[ V = (b - 2h) (a - 1.1 \text{ hour}) \times 6 \], where: \( V \) = internal left ventricular volume, \( a = \text{major axis dimension (mm)} \), \( b = \text{minor axis dimension (mm)} \), and \( h = \text{free wall thickness (mm)} \). The area of the left ventricle pressure-volume work loops was integrated to yield LV stroke work. Systolic function was evaluated using the preload recruitable stroke work relationship.21,22

**Evaluation of Neutrophil Accumulation**

Sections of left ventricular muscle were obtained from RSR13-BC animals and BC animals. Samples of left ventricle and area at risk were weighed and immediately frozen in liquid nitrogen until assayed. Samples were placed in 2 volumes of homogenization buffer (50 mmol/L sodium phosphate, pH 6.0) and homogenized (4 x 10 s at setting 5) with a Polytron homogenizer (Tekmar Co). The homogenates were centrifuged for 30 minutes (3000 g) and the supernatants removed. Myeloperoxidase (MPO) activity, a measure of neutrophil accumulation, was determined by measuring the change in absorbance at 460 nm, resulting from the conversion of \( \text{H}_2 \text{O}_2 \) in the presence of \( \text{o-dianisidine (Sigma)} \) as described previously.23 The MPO activity was normalized to the weight of the tissue sample.

**Determination of ATP, Pyruvate, and Lactate**

Myocardial ATP, pyruvate, and lactate content were measured using kits (procedures 366-A, 726-UV, and 826-UV, respectively) purchased from Sigma Diagnostics. ATP measurement was based on the conversion of 3-phosphoglyceric to glyceraldehyde-3-P. Pyruvate formation was determined by the formation of NAD; the quantification of lactate was based on the increased absorbance at 340 nm resulting from the formation of NADH.

**Histological Analysis**

Tissue morphology and cellular ultrastructure were assessed, respectively, by light and electron microscopy. Tissues from each animal were prepared for light microscopy using standard procedures. Samples of left ventricle were preserved in 10% phosphate-buffered formaldehyde, embedded in paraffin, and stained with eosin and hematoxylin. For electron microscopy, tissue samples from the left ventricular myocardium from each animal were cut into 1 mm pieces and fixed in glutaraldehyde in 0.1 mol/L sodium cacodylate buffer. Samples were dehydrated in an ethanol series, embedded in EM bed-812 (Electron Microscopy Sciences) and sectioned with a Reichert ultramicrotome. Sections were observed with a Philips CM-10 electron microscope and representative micrographs from each treatment group obtained.

**Results**

**Wet/Dry Weight Ratios**

Edema formation in the left ventricle was determined at the end of the experiment by calculation of percent weight of a biopsy specimen taken from the apex of the left ventricle: [(wet weight−dry weight)/100×wet weight].

**Statistical Analysis**

Statistical analysis was performed on a personal computer (Macintosh Ici, Apple Computers) using a statistical program (Statview, Abacus Concepts Inc). Myocardial water content was compared using a 1-way ANOVA. All other comparisons were performed by 2-way ANOVA for repeated measures with significance defined as \( P<0.05 \). In the event of a significant measures F-value, Scheffe’s method was used to localize significant differences. All values are reported as mean±SEM, with \( P<0.05 \).

**TABLE 1. Effect of RSR13-BC on Functional Parameters**

<table>
<thead>
<tr>
<th>Condition</th>
<th>NSR</th>
<th>(-dP/dt)</th>
<th>(+dP/dt)</th>
<th>ESPVR</th>
<th>Water</th>
</tr>
</thead>
<tbody>
<tr>
<td>BC</td>
<td>0%</td>
<td>26.6±2.0</td>
<td>33.7±1.7</td>
<td>33±3</td>
<td>87.7±0.8</td>
</tr>
<tr>
<td>RSR13</td>
<td>100%*</td>
<td>81.1±1.6*</td>
<td>76.3±1.9*</td>
<td>63±9*</td>
<td>79.2±0.8*</td>
</tr>
</tbody>
</table>

NSR indicates normal sinus rhythm; ESPVR, end-systolic pressure-volume relationship.

* \( P<0.05 \) vs BC.
Metabolic Preservation

The metabolic preservation of the ventricle, as assessed by preservation of ATP, was also examined (Figure 4). The total ATP content was significantly greater in the RSR13-BC group when compared with BC. Results consistent with improved oxidative metabolism were noted when tissue lactate and pyruvate were analyzed. Lactate content was markedly greater in BC myocardial samples compared with RSR13-BC. In contrast, the amount of pyruvate in BC animals was less than that in RSR13-BC animals. The lactate to pyruvate ratio was significantly less in the RSR13-BC animals compared with BC. The lactate content was higher in the BC group (87.7±0.8% versus 79.2±0.8%, P<0.05) than in the RSR13-BC group, indicative of greater edema in the myocardium of animals treated with vehicle.

Both the RSR13-BC group and the BC group had greater percentage of myocardial water content as compared with samples of naive canine myocardium. Myocardial water content was higher in the BC group (87.7±0.8% versus 79.2±0.8%, P<0.05) than in the RSR13-BC group, indicative of greater edema in the myocardium of animals treated with vehicle.

Myocardial Neutrophil Accumulation

To assess the extent of neutrophil infiltration, myocardial tissue samples were obtained for the determination of tissue MPO content. As noted in Table 2, MPO activity of BC and RSR13-BC treatment groups were not significantly different.

Histopathologic Analysis of Myocardium After Cardiopulmonary Bypass

Examination of the myocardium from BC-treated animals (Figure 5A) demonstrated an increase in myofibril spacing, suggestive of interstitial edema consistent with myocardial water content postcardioplegia. Partial loss of vessel architecture was noted in tissue samples from BC-treated animals. The endothelial cell lining on the luminal surface of both large and small vessels was disrupted (Figure 5A). The appearance of hearts receiving BC displayed marked changes in ultrastructure (Figure 5B). Extensive myofibrillar damage, as seen by blurring of the Z-bands and disruption of the myofibrils, was apparent. The mitochondria were swollen with disrupted matrices and cristae. Large amorphous densities, suggestive of irreversible injury, were noted within the mitochondria.

Myocardial architecture from RSR13-BC–treated animals was well maintained with limited interstitial edema (Figure 5C). The membrane integrity of myocytes was preserved with abundant cytoplasm present. Vessel exhibited normal endothelium, although a degree of endothelial cell swelling was noted. The myofibrils exhibited slight blurring of the Z-bands with minimal intracellular edema (Figure 5D). Mitochondria were densely packed with an intact matrix and normal appearing cristae. No amorphous densities were noted.

Discussion

Although current techniques of myocardial protection using cold cardioplegia have greatly improved postoperative outcome, poor functional recovery may be encountered, notably during prolonged surgical procedures. Avoiding ischemic injury during cardiac surgery is dependent on supplying sufficient energy to meet myocardial metabolic demands placed on the ischemic tissue.17,18 Although electromechanical arrest and hypothermia have been shown to reduce myocardial metabolic demand by 95%, the myocardium still requires energy to maintain basic cellular metabolism, ionic equilibrium, and membrane integrity.17,18 Because hypothermia impairs glycolysis and energy use, anaerobic glycolysis may be inadequate to meet even the much reduced metabolic demands of the cold arrested heart.24 In light of the problems associated with the use of hypothermic cardioplegia, it would be advantageous to develop approaches designed to promote increased oxygen delivery to the jeopardized myocardium, even under hypothermic conditions.

Compounds designed to increase oxygen delivery to hypoxic tissues without affecting blood flow or oxygen demand represent a potential pharmacologic approach for the prevention of the adverse consequences associated with prolonged ischemia. RSR13, 2-[4-[[3,5-dimethylanilino carbonyl]methyl]phenoxyl]-2-methylpropionic acid, a synthetic allostere modifier of Hb, has been shown to promote the dissociation of oxygen from Hb, even under hypothermic conditions where oxygen delivery is compromised.1,25 Theoretically, improved myocyte oxygenation by RSR13 during ischemia could improve mechanical recovery during surgical procedures requiring the use of hypothermic cardioplegia. In this study, a canine model of cardiopulmonary bypass was used to examine the ability of RSR13 to enhance functional and metabolic recovery and preserved tissue morphology compared with non-RSR13 supplemented cardioplegia. Myocardial metabolic and hemodynamic parameters (load-independent indices of cardiac function) were compared before and after cardioplegia in order to assess the effect of RSR13-supplemented cardioplegia solution.

Previous strategies for increasing tissue oxygenation during periods of ischemia have included the use of perflo-
ratios. These data demonstrate that the supplementation of myocardial function following nonthermic cardiac ischemia is fully consistent with the mechanical and metabolic data, preservation in tissues obtained from RSR13-BC was marked decrease in myofibril spacing, suggestive of edema, was observed in BC-treated hearts (A). Note swelling of endothelial cells (thin arrows) lining the blood vessels. B, At ultrastructural level there was extensive myofibrillar damage (arrowhead) with swollen mitochondria containing disrupted matrices and cristae (arrow). Myocardial architecture from RSR13-BC–treated animals (C) was well maintained, with limited interstitial edema and normal endothelial cell lining (thick arrow). D, Tissue from RSR13-BC animals showed slight morphological alterations. Myofibrils exhibited slight blurring of the Z-bands with minimal intracellular edema.

Figure 5. Light and transmission electron microscopy of cardiac tissue from hearts receiving BC (A and B) and RSR13-BC (C and D). An increase in myofibril spacing, suggestive of edema, was observed in BC-treated hearts (A). Note swelling of endothelial cells (thin arrows) lining the blood vessels. B, At ultrastructural level there was extensive myofibrillar damage (arrowhead) with swollen mitochondria containing disrupted matrices and cristae (arrow). Myocardial architecture from RSR13-BC–treated animals (C) was well maintained, with limited interstitial edema and normal endothelial cell lining (thick arrow). D, Tissue from RSR13-BC animals showed slight morphological alterations. Myofibrils exhibited slight blurring of the Z-bands with minimal intracellular edema.

In this study, animals were subject to 15 minutes of 37°C global ischemia on cardiopulmonary bypass, followed by 90 minutes of hypothermic cardioplegic arrest using BC with or without RSR13 (final concentration: 1.75 mmol/L). Postischemic LV recovery was assessed by preload recruitable stroke work and preservation of tissue morphology. Ventricular function, assessed by the slope of the preload recruitable stroke work relationship and the diastolic stress-strain relationship, was improved in the RSR13-supplement cardioplegia group compared with control. In addition to preservation of functional parameters, there were marked differences in the degree of tissue injury between the experimental groups. Histological analysis with both light and electron microscopy demonstrated that in tissues from vehicle-supplemented cardioplegia animals, there was a loss of cellular architecture consistent with ischemic injury. Specifically, there was a loss of myocyte integrity, manifested by disruption of cell membranes and blurring of cross striations. In addition, there was an increase in myofibril spacing, suggestive of edema in the BC group. The magnitude of cell preservation in tissues obtained from RSR13-BC was marked and fully consistent with the mechanical and metabolic data, demonstrating the ability of RSR13 to improve recovery of myocardial function following nonthermic cardiac ischemia after hypothermic cardioplegia. This observation was further substantiated by comparison of tissue wet weight/dry weight ratios. These data demonstrate that the supplementation of BC with RSR13, provided superior myocardial protection compared with standard BC supplement with vehicle.

The effects of increased oxygen delivery to previously ischemic tissues are incompletely understood. It should be noted that although oxygen is required for tissue survival, an overabundance of oxygen may be detrimental because of the formation of oxygen-derived free radical species. Free radical production has been demonstrated to play an important role in the pathogenesis of myocardial ischemia/reperfusion injury. Thus, with the use of compounds designed to increase oxygen delivery, comes the possibility of the generation of oxygen-derived free radicals. However, studies by Mejia et al and Pagel et al have shown that the RSR13-mediated increase in oxygen release from hemoglobin does not stimulate the formation of oxygen-derived free radicals. Furthermore, Agardh et al have demonstrated in the postischemic rat brain that hyperoxic conditions do not directly increase production of hydrogen peroxide or subsequent ischemic damage.

The proposed mechanism for RSR13-mediated cardioprotection in the setting of cardiopulmonary bypass is through increased oxygen unloading to the ischemic tissues. However, participation of other possible mechanisms cannot be excluded. A possible scenario for preservation of cardiac function associated with RSR13 is increased levels of circulating catecholamines. However, Kunert et al have shown that RSR13 does not alter serum catecholamines or renin or vasopressin concentrations, thereby negating the release of these mediators as possible contributors to the protective effect associated with RSR13. Dhole-Burger et al examined the effect of RSR13 on neutrophil accumulation. The accumulation and subsequent activation of leukocytes has been implicated in mediating tissue injury during cardiopulmonary bypass. In our study, we did not note a decrease in neutrophil accumulation in the myocardium with RSR13, suggesting the attenuation of neutrophil influx does not play a role in protecting the ischemic myocardium.

Increased delivery of oxygen promotes aerobic metabolism, enhancing generation of high-energy phosphates re-
quired for maintenance of myocardial function. Recent studies have demonstrated that RSR13 preserves intracellular pH and high-energy phosphate concentrations during low-flow myocardial ischemia, presumably by improving oxygen delivery and reducing the adverse metabolic consequences of critically diminished coronary blood flow. In the present study, samples of myocardium from RSR13-treated animals were associated with increased ATP content compared with control animals. In addition, RSR13 also was associated with a decrease in the lactate-pyruvate ratio. The improvement in cardiac functional and morphological parameters noted in the RSR13-treated group coupled with the preservation of indices of metabolic function (e.g., ATP, lactate, pyruvate) suggest that the cardioprotection seen in RSR13-treated animals may be attributable to enhanced oxygen delivery.

This study demonstrated that cardioplegia supplemented with RSR13 significantly improves cardiac mechanical function, indices of oxidative metabolism, water content, and tissue morphology in a canine model of cardiopulmonary bypass designed to closely simulate the conditions of clinical cardiac surgery. The positive findings associated with RSR13-supplemented BC suggest that the approach of increasing hemoglobin oxygen dissociation may be of benefit in the setting of cardiopulmonary bypass and that the clinical use of RSR13 warrants further investigation.

Acknowledgment

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References

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