Sulodexide Attenuates Myocardial Ischemia/Reperfusion Injury and the Deposition of C-Reactive Protein in Areas of Infarction without Affecting Hemostasis

D. Adam Lauver, Erin A. Booth, Andrew J. White, Enrique Poradosu, and Benedict R. Lucchesi

University of Michigan Medical School, Department of Pharmacology, Ann Arbor, Michigan (D.A.L., E.A.B., A.J.W., B.R.L.); and Keryx Biopharmaceuticals, New York, New York (E.P.)

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ABSTRACT

Several glycosaminoglycans (GAGs) have been demonstrated to protect the ischemic heart against reperfusion injury, in part, by modulating activation of the complement cascade. The present study assessed the cardioprotective effects of sulodexide (KRX-101), a mixture of GAGs composed of 80% low-molecular mass heparin and 20% dermatan sulfate. KRX-101 differs from other GAGs (e.g., heparin) in that it has limited anticoagulant efficacy and can be administered orally. The experimental protocol was designed to determine whether KRX-101 could protect the ischemic myocardium. Anesthetized New Zealand white rabbits underwent 30 min of coronary artery occlusion. Intravenous doses of KRX-101 (0.5 mg/kg, n = 10) or drug diluent (n = 10) were administered at the end of regional ischemia and at each hour of reperfusion. Infarct size, as a percentage of the area at risk, was calculated for both groups. Myocardial infarct size was 31.3 ± 4.1% in the vehicle- and 17.3 ± 3.2% in the KRX-101-treated animals (p < 0.05 versus vehicle). Activated partial thromboplastin times determined at baseline (preischemia) and at each hour of reperfusion (n = 4) were not significantly different between vehicle- and KRX-101-treated groups (p = N.S.). Myocardial injury was further assessed by measuring serum levels of cardiac-specific troponin I. KRX-101 administration significantly reduced (p < 0.05) the serum concentration of troponin I during reperfusion. The results suggest that KRX-101 may be an effective adjunctive agent in myocardial revascularization procedures, without the risk of increased bleeding.

The restoration of blood flow to a previously ischemic region is associated with a complex series of events that lead to tissue injury greater than what is attributed to the original ischemic insult, an event referred to as reperfusion injury. The complement system is a component of the innate immune system consisting of a group of proteins found circulating in the blood. The components of the complement system act together to recognize and destroy foreign pathogens. Activation of the complement system, however, can also have adverse actions on host tissues. In the setting of myocardial reperfusion injury, the complement system represents an integral mechanism through which the ischemic tissue undergoes injury leading to cell death and necrosis (Kilgore et al., 1994; Park and Lucchesi, 1999). Previous studies provide evidence for the role of complement by demonstrating the deposition of the terminal complement complex, or the membrane attack complex (MAC), in irreversibly injured myocardial tissue (Schafer et al., 1986). It is hypothesized, therefore, that pharmacological inhibition of complement activation may be beneficial in reducing tissue injury associated with ischemia and reperfusion.

Glycosaminoglycans (GAGs) are a group of compounds reported to be of benefit to the ischemic myocardium by preserving contractile function and reducing tissue injury (Friedrichs et al., 1994; Black et al., 1995; Gralinski et al., 1996; Kilgore et al., 1998; Tanhehco et al., 1999). In addition, selective GAGs are known to possess anticomplement activity in addition to their classical roles as anticoagulants. In the following study, we focus on sulodexide (KRX-101), a purified GAG obtained from porcine mucosa composed of 80% low-molecular mass heparin and 20% dermatan sulfate. KRX-101 differs from heparin in that it has a reduced risk of bleeding and is readily absorbed upon oral administration.

ABBREVIATIONS: MAC, membrane attack complex; GAG, glycosaminoglycan; KRX-101, sulodexide; TTC, 2,3,5-triphenyltetrazolium chloride; aPTT, activated partial thromboplastin time; PRP, platelet-rich plasma; cTnI, cardiac-specific troponin I; BSA, bovine serum albumin.
Although GAGs such as heparin are better recognized for their anticoagulant effect, less is known about their anti-inflammatory actions. Identification of more efficacious GAGs, especially those with a limited capacity to alter normal hemostasis, are of potential interest to provide adjunctive therapies to be used in procedures aimed at reducing injury resulting from reperfusion.

An important aspect of complement-mediated myocardial injury that has been the focus of recent research is the role of C-reactive protein (CRP) in the activation of the complement system. More sensitive assays for the measurement of CRP have revealed that increased CRP values, even within the range previously considered normal, may be predictive of future coronary events. Once thought of as only a nonspecific indicator of systemic inflammation, recent epidemiological research indicates that CRP might be directly involved in the pathogenesis of ischemic diseases through the activation the complement system (Beranek, 1997; Du Clos, 2000; Agrawal et al., 2001). Previous studies have demonstrated that plasma CRP concentration is directly related to infarct size following ischemia/reperfusion (Barrett et al., 2002; Hirschfield and Pepys, 2003).

In the current study, the cardioprotective effects of KRX-101 were evaluated in male New Zealand white rabbits subjected to regional myocardial ischemia/reperfusion. The protocol was designed to test the ability of KRX-101 to reduce the extent of myocardial injury associated with regional ischemia and reperfusion.

**Guidelines for Animal Research.** The procedures used in this study were in agreement with the guidelines of the University of Michigan Committee on the Use and Care of Animals. The University of Michigan Unit for Laboratory Animal Medicine provides veterinary care. The University of Michigan is accredited by the American Association for Accreditation of Laboratory Animal Care, and the animal care and use program conforms to the standards in The Guide for the Care and Use of Laboratory Animals, publication number 86-23, the National Institutes of Health, Bethesda, MD.

**Surgical Preparation.** Male New Zealand white rabbits (2.2–2.6 kg) were anesthetized with a combination of xylazine (3.0 mg/kg) and ketamine (35 mg/kg) administered intramuscularly, followed by an i.v. injection of sodium pentobarbital (15 mg/kg). After insertion of an endotracheal tube, the animals were placed on positive pressure ventilation with room air. The right jugular vein was isolated and cannulated for drug administration. The right carotid artery was isolated and instrumented with a Millar catheter microtip pressure transducer (Millar Instruments Inc., Houston, TX) positioned immediately above the aortic valve to monitor aortic blood pressure. The lead II electrocardiogram was monitored throughout the experiment. A left thoracotomy and pericardiotomy were performed, followed by identification of the left anterior descending coronary artery. A silk suture (3-0; Genzyme, Cambridge, MA) was passed under the artery and around a short length of polyethylene tubing. Simultaneous downward displacement of the polyethylene tubing while applying upward traction on the suture resulted in occlusion of the coronary artery and cessation of regional myocardial blood flow. Coronary artery occlusion was maintained for 30 min after which time reperfusion was initiated by withdrawing the polyethylene tubing. Regional myocardial ischemia was verified by the presence of a zone of cyanosis in the area of distribution of the occluded vessel and by changes in the electrocardiogram consistent with the presence of transmural regional myocardial ischemia (ST-segment elevation).

**Materials and Methods**

**Experimental Protocol.** The animals were allowed to stabilize for 15 min before beginning the protocol that involved two experimental treatment groups. The respective groups received i.v. doses of KRX-101 (0.5 mg/kg, n = 10) or saline (drug diluent, n = 10) administered at the end of regional ischemia and at each hour after the start of reperfusion, excluding the end of the 4th and final hour.

**Determination of Infarct Size.** At the completion of the 4-h reperfusion period, the hearts were removed, the aorta was cannulated, and the coronary vascular bed was perfused on a Langendorff apparatus with Krebs-Henseleit buffer at constant flow rates of 30 to 32 ml/min. The hearts were perfused with buffer for 10 min to clear the vascular compartment of plasma and blood cellular elements. Fifty milliliters of a 1% solution of 2,3,5-triphenyltetrazolium chloride (TTC) in phosphate buffer (pH 7.4, 37°C) was perfused through the heart. TTC demarcates the noninfarcted myocardium within the area at risk with a brick red color, indicating the presence of a formazan precipitate resulting from reduction of TTC by dehydrogenases present in viable myocardial tissue. Irreversibly injured tissue, lacking cytosolic dehydrogenases, is unable to form the formazan precipitate and appears pale yellow. Upon completion of the TTC infusion, the left anterior descending coronary artery was ligated at the site identical to that ligated during the induction of regional myocardial ischemia. The perfusion pump was stopped, and 3 ml of a 0.25% solution of Evans blue was injected slowly through a side arm port connected to the aortic cannula. The dye was passed through the heart for 10 s to ensure its uniform tissue distribution. The presence of Evans blue was used to demarcate the left ventricular tissue that was not subjected to regional ischemia, as opposed to the risk region. The heart was removed from the perfusion apparatus and cut into transverse sections at right angles to the horizontal axis. The right ventricle, apex, and atrial tissue were discarded. Both surfaces of each tissue section were traced onto clear acetate sheets. The images were then digitized using a flatbed scanner. The areas of the normal left ventricle nonrisk region, area at risk, and infarct region were determined by calculating the number of pixels occupying each area using Adobe PhotoShop software (Adobe Systems, Mountain View, CA). Total area at risk is expressed as the percentage of the left ventricle. Infarct size is expressed as the percentage of the area at risk.

**Hematological Measurements.** Determinations of the whole-blood activated partial thromboplastin time (aPTT) and ex vivo platelet aggregation were made at baseline and repeated after the end of regional ischemia and at each hour of reperfusion. Blood (5 ml) was withdrawn from the right jugular vein cannula into a plastic syringe containing 3.7% sodium citrate as the anticoagulant [1:10 citrate to blood (v/v)] for aPTT and ex vivo platelet aggregation determinations. Two milliliters of blood were used in the determination of aPTT using a Hemochron analyzer (Technidyne, Edison, NJ) with the reagents supplied by the manufacturer. Platelet-rich plasma (PRP), the supernatant present after centrifugation of the remaining 3 ml of whole blood at 660 rpm for 10 min, was diluted with saline-buffered saline to achieve a platelet count of 200,000 cells/ml. Platelet-poor plasma was prepared by centrifuging the remaining blood at 12,000g for 10 min and discarding the bottom cellular layer. Ex vivo platelet aggregation was assessed by established spectrophotometric methods with the use of a four-channel aggregometer (BioData PAP-4; BioData Corp., Horsham, PA) by recording the increase in light transmission through a stirred suspension of PRP maintained at 37°C. Aggregation was induced with arachidonic acid (0.65 mM) or γ-thrombin (25 nM). A subaggregatory concentration of epinephrine (550 nM) was used to prime the platelets before addition of the agonists to induce platelet aggregation. Values for platelet aggregation are expressed as percentage of light transmission standardized to PRP and platelet-poor plasma samples yielding 0 and 100% light transmission, respectively.

**Biochemical Markers of Myocardial Damage.** Plasma concentrations of cardiac-specific troponin I (cTnI) were determined by enzyme-linked immunosorbent assays (developed in conjunction
with Dr. Chris Chadwick, Life Diagnostic, Inc. West Chester, PA). Briefly, serum was prepared from whole blood drawn at baseline, after ischemia and each hour of reperfusion. Samples were frozen immediately in liquid nitrogen and stored at −80°C. On the day of the assay, samples were thawed over ice and diluted appropriately with sample diluent supplied with each assay kit. Protein concentrations were determined using the OD of each sample as compared with a standard curve.

**Immunofluorescent Detection of the Membrane Attack Complex and C-Reactive Protein.** Tissue samples used for infarct size determination were fixed in 10% buffered formalin immediately after the completion of the experimental protocol. The tissue samples were embedded in paraffin blocks and cut into sections of 2-μm thickness, which were mounted on glass slides. Two consecutive sections (mirror images) from a single heart slice were mounted on each slide. The slides were deparaffinized in three washes of xylene and rehydrated in an ethanol gradient. To remove any residual formalin and paraffin, antigen unmasking was performed using a commercially available antigen unmasking solution (Vector Laboratories, Burlingame, CA) and a pressure cooker (Fagor America, Inc., Lyndhurst, NJ). Briefly, slides were placed in a boiling solution of the unmasking agent and water. The pressure cooker was sealed, and the slides were incubated for 1 min once the cooker reached maximal pressure (approximately 15 psi). The slides were cooled immediately in a tap water bath and blocked with 3% immunohistochemical grade bovine serum albumin (BSA) for 30 min. Primary antibodies were then incubated at room temperature in a humidity chamber for 45 min.

One section per slide was incubated with a chicken anti-rabbit CRP antibody (5 μg/ml final concentration; Immunology Consultants Laboratory, Inc., Newberg, OR). The other section was incubated with a chicken anti-rabbit MAC antibody (1:2500 final dilution; Immunology Consultants Laboratory, Inc., Newberg, OR). The other section was incubated with a chicken anti-rabbit MAC antibody (5 μg/ml final; Vector Laboratories) for 30 min. Following three additional BSA washes, each section was incubated with a chicken anti-rabbit-RCR antibody (1:2500 final dilution; Immunology Consultants Laboratory, Inc., Newberg, OR). The other section was incubated with a chicken anti-rabbit MAC antibody (1:2500 final dilution; Vector Laboratories) for 30 min. Following three additional BSA washes, each section was incubated with a chicken anti-rabbit-RCR antibody (1:2500 final dilution; Immunology Consultants Laboratory, Inc., Newberg, OR).

**Staining Protocol.** The fluorescent streptavidin reagents were allowed to incubate for 30 min. After three additional BSA washes, each section was incubated with a biotinylated secondary antibody (goat anti-chicken, 1.5 μg/ml final; Vector Laboratories) for 30 min. Following three additional BSA washes, the slides were incubated with fluorescein and Texas Red (CRP and MAC sections, respectively)-labeled streptavidin (Fluorescent Streptavidin Kit; Vector Laboratories) to visualize the proteins. The fluorescent streptavidin reagents were allowed to incubate for 10 min.

**Labeling.** ProLong Gold antifade (Molecular Probes, Eugene, Oregon) and cover slips were used to preserve the sections. For comparison, digital images were captured using a digital camera (Sony DRC5000; Sony Corporation of America, New York, New York) connected to a Leica fluorescent stereoscope (Leica MZ FLIII) and the accompanying software (Leica, Wetzlar, Germany). Images were analyzed using IP Lab (Scanalytics, Inc., Fairfax, VA) software to determine mean fluorescence intensity per heart section. The sections were normalized to the amount of background on each slide. The mean intensities for three hearts in each treatment group were averaged and compared.

**Statistical Analysis.** Results are expressed as mean values ± S.E.M. Parameters between the two groups were compared using the Student’s t test for unpaired comparisons. P values <0.05 and <0.01 are regarded as significant and denoted by an asterisk and double asterisk, respectively.

**Results**

**Cardiac Contractile Parameters.** Hemodynamic variables were obtained to determine the effects of KRX-101 in mediating alterations in arterial blood pressure and heart rate. The rate-pressure product, defined as mean arterial blood pressure multiplied by the heart rate divided by 100, was used as an indicator of myocardial oxygen consumption. As depicted in Fig. 1, the rate-pressure product decreased slightly in each group (KRX-101 and vehicle) after equilibration and then remained stable throughout the duration of the protocol. The Lead II electrocardiogram did not detect any changes upon administration of KRX-101 or vehicle. Sustained ST segment elevation was observed in all rabbits during regional myocardial ischemia; however, these changes returned toward baseline upon reperfusion. No deaths from either cardiac arrhythmias or pump failure were noted in any of the groups.

**Effect of KRX-101 on Myocardial Infarct Size.** Each treatment group consisted of 10 animals in which either KRX-101 or the control vehicle was administered at the end of regional ischemia and at each hour of reperfusion, excluding the end of the 4th and final hour. The size of the area at risk expressed as a percentage of the total left ventricle was similar in each of the groups. Rabbits treated with KRX-101 exhibited significantly smaller infarcts expressed as a per-
percentage of the area at risk compared with rabbits treated with vehicle (Fig. 2).

**Blood Coagulation Parameters.** The aPTT determinations were similar in both vehicle and KRX-101 treatment groups at baseline, postischemia and each hour of reperfusion (Fig. 3). Percentage of platelet aggregation response did not change throughout the course of the experiment in the control group. Although platelet aggregation in responses to arachidonic acid remained unchanged, aggregation induced by γ-thrombin was reduced significantly in PRP prepared from blood obtained from rabbits treated with KRX-101 (Fig. 4).

**Plasma Concentrations of Biochemical Markers of Cardiac Damage.** Serum concentrations of cTnI were similar at baseline and immediately after reperfusion. KRX-101-treated rabbits exhibited significantly lower values cTnI at 1, 2, 3, and 4 h after the onset of reperfusion as compared with vehicle controls (Fig. 5).

**Immunofluorescence.** Left ventricular tissue sections used for immunofluorescence were taken from hearts that had been subjected to 30 min of regional ischemia, followed by 4 h of reperfusion. Hearts from animals treated with the drug vehicle demonstrated bright fluorescence with both anti-CRP and anti-MAC antibodies, indicating the deposition of both proteins in the area of infarction. Conversely, hearts treated with KRX-101 exhibited reduced fluorescence and, therefore, a reduction in the deposition of CRP and MAC (Fig. 6, A–D). The mean intensity of fluorescence for hearts treated with KRX-101 was significantly (p < 0.05) lower than vehicle for both CRP- and MAC-stained sections (Fig. 6E).

**Discussion**

It is understood that heparin and other GAGs have therapeutic uses beyond their tradition role as anticoagulants (Rajtar et al., 1993). In this study, we demonstrated that the i.v. administration of KRX-101 results in a decrease in myocardial infarct size in a model of in vivo regional ischemia/reperfusion. The results are in accordance with previous studies in which it was found that other glycosaminoglycans have the ability to reduce infarct size in vivo (Friedrichs et al., 1994; Black et al., 1995; Gralinski et al., 1996; Kilgore et al., 1998; Tanhehco et al., 1999).

The chemical composition of KRX-101 is defined as 80% low-molecular mass (7000 D) heparin fraction and 20% dermatan sulfate. Low-molecular mass heparin contains the same dimeric components as unfractionated heparin but has a lower degree of sulfation and shorter polysaccharide chain length. Dermatan sulfate is a polysaccharide made up of many various disaccharide units with a mean molecular mass of 25,000 D. As a result of the presence of both fractions, KRX-101 potentiates the antiprotease activities of both antithrombin III and heparin cofactor II simultaneously. Although structurally similar, KRX-101 has major differences from unfractionated heparin including prolonged half-life, reduced effect on global coagulation, and oral bioavailability (Callas et al., 1993; Buchanan et al., 1994). Low-molecular mass heparin has previously been showed to reduce infarct size following ischemia and reperfusion (Gralinski et al., 1997; Libersan et al., 1998); however, dermatan sulfate has yet to be individually investigated.

Due to their ability to inhibit the complement cascade, it is hypothesized that GAGs may prevent the adverse events associated with complement activation associated with ischemia/reperfusion. Activation of the complement cascade leads to the assembly of the MAC on cell membranes. Deposition of a sufficient number of MAC molecules on a target cell results in the disruption of the cell membrane and ultimately cell lysis.

Intravenous administration of 0.5 mg/kg KRX-101 immediately after an ischemic period and at hourly intervals during reperfusion was associated with a significant decrease in myocardial infarct size expressed as a percentage of the area at risk when compared with vehicle-treated control rabbits. The area at risk in hearts from both treated and control groups was not statistically different, thereby demonstrating that a similar amount of myocardial tissue was subjected to regional ischemia, allowing a valid comparison of infarct size between the two groups when expressed as a percentage of the risk region. Hemodynamic parameters including the rate-pressure product were similar between groups, suggest-
ing that KRX-101 is not acting to protect the myocardium by decreasing the myocardial demand for oxygen. These data indicate that differences in the myocardial oxygen demand did not contribute to protecting the myocardium after ischemia/reperfusion.

Analysis of platelet reactivity, as determined by ex vivo platelet aggregation, demonstrated a decrease in γ-thrombin-induced platelet aggregation in KRX-101-treated animals as compared with vehicle-treated animals. These data agree with previously reported findings (Rajtar et al., 1993; Cerletti et al., 1994) that indicated that KRX-101 inhibits thrombin-induced platelet activation. Interestingly, evaluation of the effects of KRX-101 on coagulation, using the aPTT, demonstrated that at the dose used in our study, there was little or no change in hemostasis.

As another method of quantifying cardiac injury after ischemia/reperfusion, we measured the serum concentration of a biochemical marker of tissue injury, cTnI. cTnI is a component of the contractile machinery within myocytes. Upon cell lysis, the protein is released into the blood and can be measured using a specific immunoassay. As would be predicted based on infarct size data, it was found that KRX-101 significantly reduced the concentration of this marker during reperfusion.

Along with other GAGs, the precise mechanism by which KRX-101 achieves myocardial protection after ischemia/reperfusion has yet to be determined. Since KRX-101 affects several aspects of reperfusion injury, various hypotheses may be drawn concerning its role in cardioprotection. Previous studies have indicated that GAGs are effective inhibitors of the complement system. Therefore, we sought to investigate the likelihood that KRX-101 acts to protect the myocardium through inhibition of the complement cascade.

CRP is an acute phase protein that has been demonstrated to be a highly sensitive, but nonspecific, marker of inflammation. Not only are plasma levels of CRP elevated in inflammatory diseases, increased CRP concentrations are associated with increased mortality due to cardiovascular events (de Beer et al., 1982; Yeh et al., 2001). Thus, CRP may be an indicator of myocardial injury, as well as being involved in the pathogenesis of irreversible myocardial injury (de Beer et al., 1982; Ridker et al., 1998; Westhuyzen and Healy, 2000; Yeh et al., 2001). The proposed mechanism of CRP involve-
The presence of the MAC in infarcted myocardium, together with the ability of inhibitors of complement to protect the ischemic myocardium, underscore the importance of pharmacologic agents capable of inhibiting the complement system in the modulation of reperfusion injury. This study also provides evidence that there is a clear separation between the anticoagulant and anticomplement effects of glycosaminoglycans. We show that a dose of KRX-101 that was previously found to have little or no effect on coagulation (Harenberg, 1998) retains the ability to protect the ischemic myocardium. Therefore, KRX-101 may be a useful agent in the reduction of ischemia/reperfusion injury with a reduced risk of adverse effects on hemostasis.

References


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Fig. 6. Representative fluorescent images of a heart from a control animal (A and C) and an animal treated with KRX-101 (B and D) after 30 min of ischemia and 4 h of reperfusion. In the vehicle-treated animal, staining for CRP (A) and MAC (C) is present in areas of infarction. In KRX-101-treated hearts, little or no staining for CRP (B) or MAC (D) can be observed in areas of infarction. E, graph illustrating the comparison of mean fluorescence intensity per heart section. Values are presented as mean ± S.E.M.; vehicle group, n = 3 (white bars); KRX-101 group, n = 3 (black bars); *p < 0.05 versus vehicle control.


Address correspondence to: Dr. Benedict R. Lucchesi, Department of Pharmacology, University of Michigan Medical School, 1301 MSRB III, Ann Arbor, MI 48109. E-mail: benluc@umich.edu