Effects of Heparin and N-Acetyl Heparin on Ischemia/Reperfusion-Induced Alterations in Myocardial Function in the Rabbit Isolated Heart

Gregory S. Friedrichs, Kenneth S. Kilgore, Peter J. Manley, Michael R. Gralinski, Benedict R. Lucchesi

Abstract  Evidence is presented that heparin pretreatment produces protective effects on myocardial tissue distinct from its anticoagulant activity. The present study examines the ability of heparin sulfate and N-acetyl heparin (a derivative of heparin devoid of anticoagulant effects) to protect the heart from injury associated with global ischemia and reperfusion. Male New Zealand White rabbits were administered either heparin sulfate (n=7, 300 U/kg IV), N-acetyl heparin (n=6, 1.73 mg/kg IV), or vehicle (n=6). Two hours after treatment, the hearts were removed, perfused on a Langendorff apparatus, and subjected to 30 minutes of global ischemia, followed by 45 minutes of reperfusion. During reperfusion, creatine kinase concentrations in the coronary sinus effluent were greater in hearts from vehicle-treated rabbits compared with hearts from N-acetyl heparin–treated and heparin-treated rabbits. Left ventricular end-diastolic pressure after 45 minutes of reperfusion in the vehicle-treated group was 64±15 mm Hg compared with 17±4 and 10±3 mm Hg in the heparin-pretreated and N-acetyl heparin–pretreated groups, respectively. Heparin, but not N-acetyl heparin, increased the activated partial thromboplastin time, consistent with its known anticoagulant action. Heparin and N-acetyl heparin inhibited complement-mediated erythrocyte lysis in a concentration-dependent manner. The glycosaminoglycans, in contrast to r-hirudin, reduced complement activation-induced injury in the rabbit isolated heart. The results demonstrate that heparin or N-acetyl heparin, administered to the intact rabbit, protects the isolated heart from subsequent myocardial dysfunction secondary to ischemia/reperfusion. The cardioprotective effects of heparin and N-acetyl heparin are independent of an antithrombin mechanism. (Circ Res. 1994;75:701-710.)

Key Words  • global ischemia • creatine kinase • tissue calcium • glycosaminoglycans • complement-mediated injury

Heparin is known to possess effects independent of its anticoagulant activity, which include inhibition of vascular smooth muscle cell proliferation,1-3 inhibition of leukocyte-mediated damage,4,5 and release of lipoprotein lipase from cardiac myocytes.6 Other important and often unrecognized actions of heparin and other glycosaminoglycans are their ability to inhibit complement activation7 and to exhibit anti-inflammatory effects.8-10 The ability of heparin and its derivatives to produce effects beyond hemostasis has not received much attention despite data suggesting that heparin protects skeletal11 and cardiac muscle12 subjected to ischemia/reperfusion.

The impetus for this investigation originates from the common use of heparin in many ischemia/reperfusion studies. For example, a recent publication13 reported significant qualitative differences in the development of myocardial contracture between blood-perfused and crystalloid-perfused rat hearts subjected to comparable protocols involving ischemia and reperfusion. The blood-perfused hearts displayed improved recovery of function after cessation of ischemia when compared with crystalloid-perfused hearts. A difference between the two groups was the continuous presence of heparin in the protocol involving the perfusion of hearts with whole blood and the absence of heparin in the crystalloid-perfused hearts.

There is evidence implicating complement-mediated damage in a variety of tissues subjected to conditions of ischemia/reperfusion14-19 as well as in response to the direct cytotoxic actions resulting from complement activation.20 The ability of heparin and N-acetyl heparin to inhibit selected components of the complement cascade has been documented.9,21,22 Therefore, we hypothesized that if complement is involved in myocardial dysfunction caused by ischemia/reperfusion, then pretreatment with heparin would improve the myocardial dysfunction resulting from the ischemic insult. To distinguish between the antithrombin and anticomplement actions of heparin, we examined N-acetyl heparin, which is devoid of antithrombin activity, whereas both heparin and N-acetyl heparin share the common property of inhibiting complement activation.21

The primary goal of the present study was to determine whether heparin and N-acetyl heparin administered in vivo could attenuate functional deterioration produced in the isolated heart subjected to global ischemia and reperfusion. Membrane damage produced as the result of ischemic injury was assessed via changes in hemodynamic variables, creatine kinase (CK) re-
lease, and changes in total tissue calcium. The results of this investigation demonstrate that both heparin and N-acetyl heparin have the capability to reduce the extent of contractile dysfunction and cellular alterations associated with ischemia/reperfusion. The cardioprotective effects of heparin and N-acetyl heparin are unrelated to changes in hemostatic mechanisms and may involve the prevention of tissue complement activation.

Materials and Methods

Guidelines for Animal Research

The procedures used in the present study are in accordance with the guidelines of 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 the Accreditation of Laboratory Animal Care. The animal care and use program conforms to the standards in “The Guide for the Care and Use of Laboratory Animals,” Department of Health, Education, and Welfare Publication No. (NIH) 86-23.

Isolated Heart Preparation

Venous blood samples from a lateral ear vein (1.5 mL) were obtained from conscious New Zealand White rabbits (1.8 to 2.0 kg) for determination of the activated partial thromboplastin time (aPTT). The test was performed by using a Hemo-chron 801 whole-blood coagulation system and reagents for the OneStep aPTT (International Technidyne Corp). The aPTT is a quantitative whole-blood coagulation test of the intrinsic coagulation pathway used to monitor the status of heparin anticoagulation. OneStep aPTT uses a kaolin activator to initiate the coagulation process and a platelet factor 3 substitute. The reagent system provides linearity to heparin up to 1.5 U/mL of blood, allowing aPTT results to be reported up to a plasma equivalent of 300 seconds for whole blood.

Rabbits were administered either heparin (300 U/kg IV, 185.5 USP mL/mg), N-acetyl heparin (1.73 mg/kg IV), or vehicle (0.3 mL/kg IV, consisting of physiological saline containing 0.003% benzyl alcohol) and returned to their cages. A second venous blood sample was withdrawn 2 hours later, and the aPTT determination was repeated. Immediately thereafter, the rabbits were euthanized by cervical dislocation. A median sternotomy was performed rapidly, and the heart was removed. The aorta was cannulated (Langendorff preparation) and perfused at a constant rate of 18 to 22 mL/min with a buffer solution. The isolated hearts were allowed to stabilize for 20 minutes. Once a steady state was established, the rate of coronary flow was maintained constant during periods of perfusion. The buffer perfusate (pH 7.4) was of the following composition (mmol/L): NaCl 117, KCl 4.0, CaCl₂·2H₂O 2.4, MgCl₂·6H₂O 1.2, NaHCO₃ 25, KH₄PO₄ 1.1, glucose 5.0, l-glutamate (sodium salt) 5.0, and sodium pyruvate 2.0. The perfusate was passed through a "membranous lung" fabricated from 6 m of medical-grade gas-permeable tubing (Silastic. Dow Corning Corp) placed in a double-walled water-heated chamber. The chamber was gassed continuously with 95% O₂/5% CO₂. The temperature of the buffer medium and isolated heart was maintained at 37°C with a temperature-controlled circulating water bath. The hearts were paced electrically from the right atrium throughout each protocol at a frequency 10% to 20% above the intrinsic rate. A latex balloon was advanced into the left ventricle through the left atrial appendage. The balloon was expanded to establish a left ventricular end-diastolic pressure (LVPed) of 5 mm Hg. The intraventricular balloon was attached to a pressure transducer via a rigid cannula. The pressure transducer was positioned at the level of the heart and for simultaneous and continuous measurement of left ventricular developed pressure (LVDP) and LVPed. A polyethylene cannula was placed in the left ventricle to vent the chamber. A thermistor probe (Tele-thermometer, Yellow Springs Instrument Co) was placed in the left ventricle and served to monitor heart temperature throughout the protocol. The electrogram was recorded from bipolar leads positioned between the aorta and cardiac apex.

All variables were recorded with a Grass polygraph (model 7, Grass Instrument Co) and included the electrogram, coronary perfusion pressure (CPP), left ventricular systolic pressure (LVPPs), and LVPed. LVDP is equal to LVPPs minus LVPed.

Experimental Protocol

After 15 to 20 minutes of stabilization, the volume of the perfusion medium in the system was reduced to 300 mL, and recirculation of the perfusate was begun. After a 5-minute period of recirculation, global myocardial ischemia was induced by stopping the perfusion pump, thereby abruptly arresting coronary perfusion. A constant heart temperature of 37°C was maintained throughout the period of global ischemia. After an elapsed period of 30 minutes, perfusion of the heart was resumed at the preestablished rate of coronary flow and continued for 45 minutes.

The study population consisted of three experimental groups in which the hearts underwent a 30-minute period of global ischemia, followed by 45 minutes of reperfusion as described above. All treatment regimens were conducted by administering the following selected agents to the conscious rabbit: group I (n=7), in vivo heparin (300 U/kg IV) pretreatment; group II, (n=6) in vivo N-acetyl heparin (1.73 mg/kg IV); and group III (n=6), in vivo vehicle pretreatment. Hemodynamic variables were recorded every 10 minutes for the entire 45-minute reperfusion period. A second series of animals pretreated with either heparin (300 U/kg IV, n=3) or N-acetyl heparin (1.73 mg/kg IV, n=3) for 60 minutes was added to explore the time-dependent effect of pretreatment with either agent.

CK Assay

Coronary venous effluent (500 μL) was collected from the pulmonary artery at preselected time intervals (baseline and 10, 20, 30, and 45 minutes of reperfusion) for determination of CK levels. Samples were frozen immediately in liquid nitrogen and stored for later analysis. A standard spectrophotometric assay (Sigma Diagnostics, procedure No. 47-UV) was used for these determinations.^

Tissue Calcium Ion Determinations

Hearts were frozen in liquid nitrogen immediately after completion of the protocol and stored for later analysis. Myocardial tissue specimens (2 g) from the left ventricular free wall were dissolved in 70% HNO₃ and adjusted to pH 5.0 (with 15 mol/L NaOH). A calorimetric assay for calcium (Arsenazo III) was used to determine tissue calcium content (Sigma Diagnostics, procedure No. 588).

Assessment of Complement Inhibition Using Rabbit Plasma and Human Erythrocyte Lysis

Complement-mediated red blood cell hemolysis was assessed by a turbidometric technique similar to that described previously. Human blood (2 mL) was collected and centrifuged at 2000g for 10 minutes at room temperature. The plasma layer was discarded, and the red blood cells were washed three times with phosphate-buffered saline (PBS, pH 7.4). A solution of 10% erythrocytes was prepared in assay buffer (PBS containing 0.25% bovine serum albumin, pH 7.4). The assay for detection of hemolysis was performed with a PAP-4 platelet aggregometer (Bio/Data Corp) by using microvolume cuvettes and a stir speed of 1200 rpm. One hundred percent light transmission was defined by use of a solution of 0.5% red blood cells lysed with whole rabbit plasma. Whole...
plasma (285 μL) and the agents (15 μL) to be tested were added to the cuvette and warmed to 37°C. The assay was initiated by the addition of 15 μL of the 10% red blood cell suspension (0.5% final dilution), bringing the total volume in the cuvette to 315 μL. Red blood cell lysis resulted in an increase in light transmission that was monitored for 5 minutes. Five sets of experiments were performed: (1) vehicle treatment plus human erythrocytes plus rabbit plasma, (2) heparin treatment (0.32, 0.64, and 1.28 mg/mL) plus human erythrocytes plus rabbit plasma, and (3) N-acetyl heparin treatment (0.32, 0.64, and 1.28 mg/mL) plus human erythrocytes plus rabbit plasma. Additional groups included (4) soluble complement receptor 1 (sCR1, 5.0, 16.7, and 50 nmol/L) plus human red blood cells plus rabbit plasma and (5) heat-inactivated (50°C for 40 minutes) rabbit plasma plus human erythrocytes. Concentrations described reflect total cuvette concentration of each test agent.

Assessment of Complement Inhibition Using Human Plasma and Rabbit Erythrocyte Lysis

An additional cell lysis protocol was performed by substituting rabbit erythrocytes for human red blood cells in the previous assay and human plasma for rabbit plasma. Human plasma was diluted 1:10 with PBS and used to lyse a 10% rabbit red blood cell suspension in the presence of graded concentrations of heparin (0.03, 0.128, and 0.32 mg/mL) and N-acetyl heparin (0.128, 0.32, and 1.28 mg/mL).

Assessment of Complement Inhibition Using Human Plasma and the Perfused Rabbit Heart

The rationale for the use of human plasma for induction of complement-mediated tissue injury derives from the studies of Platts-Mills and Ishizaka27 demonstrating that human serum caused the hemolysis of unsensitized rabbit red blood cells as a result of activation of the alternative pathway of complement. The ability of rabbit lung tissue to activate the human complement system was demonstrated by Seeger et al.26 Recent studies by Homeister and colleagues20,29 demonstrated complement-mediated injury when the rabbit isolated heart was perfused in the presence of human plasma. If heparin, N-acetyl heparin, or r-hirudin was capable of attenuating complement-mediated myocardial injury, then exposure to human plasma in the presence of either agent would not be expected to produce deleterious effects in the rabbit isolated heart. In this protocol, four groups of hearts were studied. Each group consisted of three isolated hearts in which 4% human plasma was added to the perfusion medium and allowed to circulate for 30 minutes as described previously.20,29 Four separate treatment regimens were used: (1) a control group in which placebo diluent was added to the perfusion medium, (2) a group in which the hearts were treated in vitro with heparin (100 U/mL) added to the perfusion medium 5 minutes before the addition of 4% human plasma, (3) a group of hearts pretreated in vitro with the addition of N-acetyl heparin (0.6 mg/mL) added to the perfusion medium 5 minutes before the addition of 4% human plasma, and (4) a group of hearts pretreated in vitro with the addition of r-hirudin (10.0 mg/mL) added to the perfusion medium 5 minutes before the addition of 4% human plasma. LVPed, LVVps, and CPP were recorded continuously throughout the protocol in which the hearts were observed for 30 minutes after addition of 4% human plasma to the perfusion medium.

Drugs and Reagents

Heparin sodium injection (1000 U/mL) was obtained from Elkins-Sinn, Inc. r-Hirudin was obtained from CIBA-GEIGY, and N-acetyl heparin (molecular weight, 12,000 to 16,000) was purchased from Sigma Chemical Co. Analytical grade chemicals used for preparation of the perfusate solution were obtained from commercial sources. The investigational supply of sCR1 was a gift from Smith-Kline Beecham.

After informed consent was obtained, venous blood was collected from fasted human volunteers. The blood (60 mL) was placed into tubes containing heparin (final concentration, 14 U/mL) and mixed. The concentration of heparin used has been shown not to inhibit complement activation. The blood was centrifuged at 2000g for 10 minutes, and the plasma was collected and stored at 20°C until use (not longer than 48 hours). Immediately before use, the plasma was filtered through a C-18 cartridge (Millipore). Rabbit blood was obtained from the marginal ear vein and treated in the same manner as described for collection and preparation of the human plasma.

Statistical Analysis

Statistical analyses were performed with a MacIntosh Computer and SUPERANOVA software (Abacus Concepts, Inc). The data are expressed as mean±SEM. A repeated-measures ANOVA (one factor within, one between) was performed to test the interaction between time and treatment and the overall effects of groups and time. A one-factor ANOVA was performed within groups at appropriate time points to assess the effects of time. A one-way ANOVA (factorial) was performed between treatment groups to determine where differences existed at specified times. Differences were considered significant at P<.05.

Results

Whole-Blood aPTT

Whole-blood aPTT was determined before and after in vivo administration of heparin, N-acetyl heparin, or vehicle to the intact rabbit. The aPTT is a quantitative whole-blood coagulation test of the intrinsic coagulation pathway that is influenced by the degree of heparin anticoagulation.30 The mean baseline aPTT value for rabbits in the control group was 34±2 seconds (n=6). Two hours after the administration of vehicle to each of the control animals, the mean aPTT was 34±2 seconds, a value that did not differ from the initial determination. In heparin-treated rabbits (n=7), aPTT increased from a mean baseline value of 35±3 to 60±6 seconds (P<.05 compared with baseline). When N-acetyl heparin (n=6) was administered in an amount comparable to heparin sulfate administration (in milligrams per kilogram), it failed to alter the aPTT when determined 2 hours after administration of the acetylated glycosaminoglycan (from 44±2 seconds [baseline] to 49±3 seconds [after treatment]). Thus, N-acetyl heparin, unlike heparin, did not affect the intrinsic pathway associated with the blood coagulation mechanism.

CK Release in Pulmonary Effluent From Ischemic/Reperfused Rabbit Hearts

CK release from hearts of heparin-pretreated or N-acetyl heparin–pretreated rabbits was reduced significantly compared with hearts from vehicle-treated rabbits (Fig 1). There were no differences in baseline CK among groups. However, there was a significant difference in total CK release when hearts from animals treated with heparin or N-acetyl heparin were compared with control hearts. Twenty minutes after the onset of reperfusion, CK release from hearts of vehicle-treated rabbits was 451±111 U/L compared with 171±20 U/L from hearts of heparin-pretreated rabbits and 48±22 U/L from hearts of N-acetyl heparin–pretreated rabbits (each P<.05 versus vehicle-treated hearts). At each subsequent time point throughout the period of reperfusion, hearts from vehicle-treated rab-
from the groups pretreated with N-acetyl heparin and with heparin. LVPPs throughout reperfusion averaged 75±4 and 86±2 mm Hg in hearts from rabbits pretreated with heparin and N-acetyl heparin, respectively, whereas hearts from vehicle-pretreated rabbits averaged 104±12 mm Hg (P<.05). The apparent increase in the LVPPs is a reflection of the upward shift in the LVPed. The values for LVPPs and LVPed for the respective groups are presented graphically in Fig 2.

LVPed was set initially at a value of 5 mm Hg in all hearts by adjusting the volume in the fluid-filled intraventricular balloon. LVPed, an arbiter of ischemic contracture and reperfusion injury, was elevated markedly 10 and 45 minutes after initiating reperfusion in hearts from vehicle-pretreated rabbits. On reperfusion, LVPed increased abruptly and after 10 minutes was 69±14 mm Hg, with only a slight decline to 64±15 mm Hg after 45 minutes of reperfusion. The hearts from heparin-pretreated rabbits developed an abrupt increase in LVPed on reperfusion, reaching a value of 43±11 mm Hg at 10 minutes but declining rapidly to 17±4 mm Hg by the end of 45 minutes into the reperfusion period. The cardioprotective effect of N-acetyl heparin was evident on reperfusion. The initial value for LVPed 10 minutes after reperfusion was 14±3 mm Hg, and after 45 minutes of reperfusion, LVPed had stabilized at 10 mm Hg. The data for LVPed are shown graphically in Fig 3. The increased LVPed was sustained throughout the period of reperfusion in the control group, whereas hearts from heparin-pretreated or N-acetyl heparin–treated rabbits had an increase in LVPed at the start of reperfusion but recovered progressively during reperfusion so that end-diastolic function was improved significantly over that of the control hearts. The onset of global ischemia is accompanied by a decline in developed pressure to 0 mm Hg in each of the three groups, with an abrupt rise in diastolic tension on reperfusion. The increase in the LVPed on reperfusion represents a loss in left ventricular compliance and tends to increase the absolute value of LVPPs as progressive contracture raises the pressure within the ventricular balloon. The difference between the LVPPs and the LVPed represents LVDP, which was decreased significantly in hearts from the vehicle-treated group. Improved diastolic compliance and an improvement in LVDP is evident in hearts from both the heparin-treated and N-acetyl heparin–treated groups.

After reperfusion, CPP, an index of vascular resistance, increased progressively. The greatest increase was observed in the hearts from the control group. CPP at the end of the experimental protocol was significantly less in the hearts from the heparin-treated and N-acetyl heparin–treated animals compared with control hearts, with improvement being most complete in the N-acetyl heparin–treated hearts. The data are presented graphically in Fig 4.

**Time Dependence for the Effects of Heparin or N-Acetyl Heparin**

Hearts from rabbits pretreated in vivo with either heparin (300 U/kg IV, n=3) or N-acetyl heparin (1.73 mg/kg IV, n=3) for 1 hour, as opposed to 2 hours, were subjected to an identical ischemia/reperfusion protocol described earlier to explore the time-dependent nature of the heparin-mediated or the N-acetyl heparin–medi-

**Tissue Calcium**

Myocardial tissue calcium concentration was determined in each heart after the completion of the protocol in which hearts were subjected to 30 minutes of global ischemic arrest followed by 45 minutes of reperfusion. Hearts from heparin-treated rabbits had a total tissue calcium concentration of 29±0.5 μmol/g, which was less than that of heart tissue from the vehicle-pretreated group, 35±1 μmol/g (P<.05). The calcium concentration of hearts from the N-acetyl heparin–treated group was 24±5 μmol/g (P<.05 versus vehicle-pretreated hearts).

In vivo treatment with the glycosaminoglycans resulted in a significant reduction in myocardial tissue calcium accumulation associated with global ischemic arrest and reperfusion.

**Hemodynamics: Global Ischemia and Reperfusion**

Global ischemia produced significant changes in the measured hemodynamic variables. On reperfusion, there was an increase in CPP, a reduction in LVPPs, an increase in LVPed, and a decline in LVDP in all groups of hearts compared with their respective baseline values. However, the degree to which these variables changed differed among groups. LVPPs in hearts of the vehicle-treated group was greater than LVPPs in hearts...
Friedrichs et al  Glycosaminoglycans and Myocardial Protection 705

Fig 2. Graphs showing left ventricular systolic pressure (LVPs) and left ventricular end-diastolic pressure (LVPed) during baseline, the 30-minute period of global ischemia, and after reperfusion for 45 minutes. A. Hearts are from vehicle-treated rabbits. B. Hearts are from heparin-treated rabbits (300 U/kg IV). C. Hearts are from N-acetyl heparin (1.73 mg/kg)-treated rabbits. Reperfusion of the isolated heart, after a 30-minute period of global ischemic arrest at 37°C, is accompanied by a sustained increase in LVPed, which is most prominent in hearts from the vehicle-treated group. LVPed in the vehicle-treated group is increased significantly after reperfusion above the initial baseline value of 5 mm Hg at all time points. Hearts from rabbits pretreated with heparin or N-acetyl heparin showed a progressive reduction in LVPed during reperfusion, with a decline toward the preischemic values. At all time points after reperfusion, the LVPed value was significantly less than the control group value at comparable time points. In vivo exposure of the heart to N-acetyl heparin provided significant protection against the effects of global ischemia and reperfusion. The LVPed value was significantly less than values for the control group and the heparin-treated group at all time points after reperfusion. Recovery of cardiac function during the early phase of reperfusion was significantly improved in the N-acetyl heparin-treated group. Although recovery of LVPed was delayed in heparin-treated hearts, compliance was improved compared with recovery in control hearts and approximated that of N-acetyl heparin-treated hearts at 30 and 45 minutes after the start of reperfusion. *P<.05 vs corresponding value for vehicle-treated group.

Fig 3. Graph showing left ventricular end-diastolic pressure (LVPed) for each of the three groups of hearts superimposed to facilitate comparison of recovery of ventricular compliance on reperfusion after a 30-minute period of global ischemic arrest. LVPed was initially established in all hearts at a value of 5 mm Hg by expanding the fluid-filled intraventricular balloon. The hearts from the vehicle-treated rabbits displayed a marked and sustained increase in resting tension (LVPed) throughout the 45 minutes of reperfusion. At each of the respective time points after the start of reperfusion, LVPed in the vehicle-treated hearts exceeded LVPed in the heparin-treated and N-acetyl heparin-treated hearts. The hearts from rabbits pretreated with either heparin or N-acetyl heparin recovered to the same extent by the end of 30 minutes after the start of reperfusion. The symbols †, *, and ‡ indicate P<.05 according to the key at the bottom of the figure.

baseline values and compared with hearts from vehicle-treated rabbits. The data are tabulated in the Table. The results are in marked contrast to what was observed with each of the glycosaminoglycans administered 2 hours before preparing the hearts for in vitro perfusion.

Inhibition of Rabbit Complement Activation: Rabbit Plasma and Human Red Blood Cell Lysis

An assay protocol was developed to determine if rabbit complement can be inhibited by heparin or N-acetyl heparin. Human erythrocytes were exposed to...
Hemodynamic Parameters After 60 Minutes of In Vivo Exposure to Heparin or N-Acetyl Heparin and Subsequent In Vitro 30-Minute Global Ischemia/Reperfusion

<table>
<thead>
<tr>
<th></th>
<th>Heparin, mm Hg</th>
<th>N-Acetyl Heparin, mm Hg</th>
<th>Vehicle, mm Hg</th>
</tr>
</thead>
<tbody>
<tr>
<td>CPP Baseline</td>
<td>60±4</td>
<td>45±1</td>
<td>50±9</td>
</tr>
<tr>
<td>Reperfusion</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20 min</td>
<td>123±41</td>
<td>77±2*</td>
<td>124±28*</td>
</tr>
<tr>
<td>45 min</td>
<td>131±40</td>
<td>90±10*</td>
<td>166±26*</td>
</tr>
<tr>
<td>LVDP Baseline</td>
<td>98±10</td>
<td>89±7</td>
<td>83±5</td>
</tr>
<tr>
<td>Reperfusion</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20 min</td>
<td>41±5*</td>
<td>29±12*</td>
<td>34±10*</td>
</tr>
<tr>
<td>45 min</td>
<td>50±7*</td>
<td>47±9*</td>
<td>41±9*</td>
</tr>
<tr>
<td>LVPed Baseline</td>
<td>4±1</td>
<td>5±1</td>
<td>5±1</td>
</tr>
<tr>
<td>Reperfusion</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20 min</td>
<td>62±4*</td>
<td>69±10*</td>
<td>66±15*</td>
</tr>
<tr>
<td>45 min</td>
<td>56±1*</td>
<td>60±13*</td>
<td>64±15*</td>
</tr>
</tbody>
</table>

CPP indicates coronary perfusion pressure; LVPed, left ventricular end-diastolic pressure; and LVDP, left ventricular developed pressure (left ventricular systolic pressure minus LVPed). Values are mean±SEM.

Heparin was at 300 U/kg IV (n=3); N-acetyl heparin, 1.73 mg/kg IV (n=3); and vehicle, 0.6 mL saline IV (n=6).

*P<.05 vs corresponding baseline value.

rabbit plasma in the absence and presence of increasing concentrations of heparin or N-acetyl heparin. Quantitative assessment of red blood cell lysis was accomplished with the use of a nephelometric method in which light transmission increases directly with the extent of red blood cell lysis. Therefore, less light transmission would occur after the inhibition of complement activation. The concentrations of heparin or N-acetyl heparin tested were 0.32, 0.64, and 1.28 mg/mL. The bioassay is dependent on the activation of rabbit plasma complement by human erythrocytes, thus leading to the formation of the membrane attack complex. The latter is responsible for the lysis of the human red blood cells. The results indicate that human red blood cell lysis is inhibited in a concentration-dependent manner by either heparin or N-acetyl heparin. All readings were performed 5 minutes after the addition of the rabbit plasma to the suspension of human erythrocytes. Heparin in a concentration of 0.32 mg/mL significantly reduced the extent of human red blood cell lysis compared with buffer treatment alone, with nearly complete inhibition (95±5%) of human red blood cell lysis at a heparin concentration of 1.28 mg/mL. Inhibition of rabbit plasma complement–induced red blood cell lysis with N-acetyl heparin treatment was significant at a concentration of 0.64 mg/mL (27±6%) when compared with the control condition (P<.05) and with further inhibition to 98±1% at a concentration of 1.28 mg/mL. Thus, the presence of either glycosaminoglycan showed a concentration-dependent inhibition of rabbit plasma complement as indicated by the inhibition of complement-mediated human red blood cell lysis.

Inhibition of Human Complement Activation: Human Plasma and Rabbit Red Blood Cell Lysis

The ability of heparin or N-acetyl heparin to inhibit human plasma complement was examined by exposing rabbit red blood cells to human plasma. Heparin (0.03, 0.128, and 0.32 mg/mL) inhibited human complement–induced lysis of rabbit red blood cells in a concentration-dependent manner. The respective concentrations of heparin resulted in 58±5%, 71±3%, and 100±0% (P<.05 versus vehicle) inhibition of rabbit red blood cell lysis. Concentrations of N-acetyl heparin that produced significant attenuation in rabbit red blood cell lysis were 0.32 and 1.28 mg/mL, representing 88±1% and 99±0% inhibition, respectively. On the basis of an in vitro assay, both heparin and N-acetyl heparin modulate activation of the rabbit and the human complement system in a concentration-dependent manner.

Inhibition by sCR1 of Rabbit Complement–Induced Human Red Blood Cell Lysis

Additional studies were performed with the red blood cell lysis assay using rabbit plasma and human red blood cells in the presence of graded concentrations of sCR1, which is known to intercept the catalytic subunit (C3b) of the C3 and C5 convertases of both the classic and alternative complement pathways. Human red blood cell lysis by rabbit plasma was inhibited by sCR1 in concentrations ranging from 5.0 to 50 nmol/L. Increasing the concentration of sCR1 to 16.7 nmol/L resulted in a significant attenuation of human red blood cell lysis, from 2±2% (buffer treated) to 73±3% (16.7 nmol/L, sCR1). A concentration of 50 nmol/L sCR1 was sufficient for complete inhibition of cell lysis (100±0%). Heat-inactivated rabbit plasma did not lyse human erythrocytes, further demonstrating the dependent role of complement activation for the cytolytic effect.

Inhibition of Complement-Mediated Injury in Rabbit Isolated Hearts by Heparin or N-Acetyl Heparin but not r-Hirudin

Fig 5 illustrates the changes in selected hemodynamic variables after exposure of the rabbit isolated heart to 4% normal human plasma for 30 minutes in the presence of exogenously added vehicle, heparin, N-acetyl heparin, or r-hirudin. Contact of the rabbit heart tissue with human plasma results in activation of the complement system27 and subsequent formation of the membrane attack complex, resulting in myocardial dysfunction.20 The addition of heparin or N-acetyl heparin to the perfusion medium significantly attenuated the deleterious effects of 4% normal human plasma on the rabbit isolated heart. Within 30 minutes of exposure to human plasma, LVPed increased from a baseline value of 6±1 to 68±5 mm Hg in the control hearts (P<.05). Within 30 minutes after addition of 4% human plasma to the perfusion medium, LVDP had declined significantly in the control group, indicative of severe cardiac dysfunction, an effect that was prevented by simultaneous perfusion of the hearts with heparin or N-acetyl heparin. However, the group of hearts exposed to r-hirudin was unprotected from the cytotoxic effects of
of its interaction with AT III. Heparin is known to exert an anti-inflammatory action and to exhibit cytoprotective activity. Intravenous administration of heparin reduced the extent of myocardial injury, as assessed by tissue CK release and myocardial necrosis, in the canine heart subjected to coronary artery occlusion. Low molecular weight heparin, enoxaparin, was reported to limit the extent of myocardial injury in the experimental animal subjected to 90 minutes of coronary artery occlusion followed by reperfusion. In the previously cited studies, the mechanism for the observed protection was not delineated.

Other recognized pharmacologic actions of heparin include the release of lipoprotein lipase, the ability to activate platelets, the regulation of angiogenesis, and the inhibition of complement activation and assembly of the terminal membrane attack complex. With respect to the latter action, it is noted that heparin and related glycosaminoglycans bind to the endothelium and increase membrane-associated heparan sulfate proteoglycans.

We compared the cardioprotective effects of commercial heparin with a structurally related derivative, N-acetyl heparin. The latter differs significantly from heparin in that it lacks anticoagulant activity but, like heparin, is able to modulate or prevent the activation of complement. The protective effects of heparin or N-acetyl heparin were apparent in isolated hearts that were exposed in vivo to the glycosaminoglycans for 2 hours and then subjected to in vitro perfusion. The enhanced protection provided by N-acetyl heparin may be attributed to its greater affinity for endothelial cells, as suggested by the observations that structural modifications of heparin show greater endothelial cell binding and reduced anticoagulant properties than the parent heparin. As demonstrated by Barzu et al, heparin binds to AT III so that the latter competes with the endothelium for binding of the glycosaminoglycan. Chemically modified nonanticoagulant heparins such as N-acetyl heparin, which lack or have a reduced affinity for AT III, may be more effective cytoprotective agents than the parent heparin when given in equivalent doses.

Compared with heparin, a greater proportion of the administered dose of N-acetyl heparin (with a lower affinity for AT III) would be bound and perhaps internalized by the endothelial cell. Although speculative at this point, the possibility exists that heparin or N-acetyl heparin exerts its cardioprotective effect in the isolated heart model via inhibition of tissue complement activation within the interstitial space or at the surface of cell membrane. Heparin and presumably its N-acetylated derivative bind avidly to the luminal surface of the endothelium and are transported to the intracellular space. Heparin is internalized and remains associated with the cell for extended periods of time, as indicated by the presence of metachromatic inclusions when stained with toluidine blue. The localization of glycosaminoglycans to the endothelium is time dependent and provides a mechanism whereby heparin or N-acetyl heparin could protect the ischemic/reperfused heart independent of its interactions with blood-based proteins or circulating blood cells. Hearts from rabbits pretreated with heparin or N-acetyl heparin for shorter periods of time (1 hour) were not protected from an in vitro ischemia/reperfusion challenge. Internalization by human plasma and did not differ from the control group with respect to the measured variables.

Exposure of the hearts to 4% human plasma resulted in a significant increase in the CPP in the control group compared with the hearts treated in vitro with either heparin or N-acetyl heparin.

**Discussion**

Heparin consists of a mixture of highly electronegative acidic mucopolysaccharides (molecular weight, 5000 to 50 000) that contain numerous N- and O-sulfate linkages. Heparin is best recognized for its ability to prevent blood coagulation by catalytically accelerating the interaction of antithrombin III (AT III) with thrombin, as well as with factors XIIa, IXa, VIIa, and Xa, thereby inhibiting the proteases necessary for completion of the coagulation cascade. There are published data to indicate that heparin possesses multiple actions that are independent
the endothelial cell and slow release of glycosaminoglycans could account for the protective effects in the isolated perfused heart studied 2 hours after the in vivo administration of heparin or N-acetyl heparin.

In addition to preserving myocardial function, heparin and N-acetyl heparin pretreatment reduced the loss of cytosolic CK and reduced the accumulation of tissue calcium. The inability of N-acetyl heparin to alter aPTT while at the same time providing a cardioprotective effect provides compelling evidence that the salutary effect of heparin is independent of thrombin inhibition.

This investigation raises a question regarding the mechanism by which the glycosaminoglycans mediate the observed protective effects against ischemia/reperfusion-induced alterations in myocardial function in an isolated heart that has been exposed to either agent in vivo. Potential mechanisms are suggested by reports describing other known effects of heparin, including inhibition of leukocyte elastase, inhibition of leukocyte activation, and displacement of extracellular superoxide dismutase, as well as anti-inflammatory effects dependent on inhibition of complement activation.5,8,9,21,24,37-46,47 The protection provided by the in vivo administration of heparin or N-acetyl heparin was observed in the isolated perfused heart, therefore requiring tissue retention of the glycosaminoglycans and their continued presence during perfusion in vitro.48 The binding of glycosaminoglycans to endothelial cells has been shown in vivo and in vitro.38-40,48 The localization of the glycosaminoglycans at the endothelial cell surface and their internalization may account for the observed sustained cardiac protective effect. The ex vivo cardioprotective effect in response to the in vivo administration of the glycosaminoglycans required that the drugs be administered 2 hours before the hearts were isolated. The delayed development of the cardioprotective effect may reflect the need for the glycosaminoglycans to become associated with the endothelial cell membrane and to gain access to an intracellular site. Localization on, or near, the cell surface would place the glycosaminoglycans in a position where complement activation and the assembly of the membrane attack complex occur, thereby allowing them to prevent activation of the complement cascade. Heparin concentrations in endothelium versus plasma are ≈ 100 times greater after intravenous or ex vivo administration of the drug.49 The protracted release from endothelial cells of previously internalized heparin has been documented.42,44 Heparin could be recovered up to 96 hours after exposure. The distribution of heparin across endothelial cell membranes is concentration and time dependent. The protracted retention of heparin intracellularly by endothelial cells and subsequent release is consistent with the observations made in the present study. Furthermore, the uptake and retention of heparin by endothelial cells would lessen the importance of plasma glycosaminoglycan concentration, which has a short duration in plasma. Only 1% of the administered heparin dose is detectable 6 minutes after administration despite the fact that the anticoagulant activity persists beyond the point at which plasma heparin is no longer measurable.49,50

Heparin and N-acetyl heparin have been implicated in the inhibition of complement activation both in vivo and in vitro, an observation that was confirmed in the present study through the use of the red blood cell lysis assay. Heparin prevents formation of the C3 convertase C3bBb, thus preventing subsequent formation of the membrane attack complex.31,36,37,51-53 The membrane attack complex has been shown to produce deleterious effects on the rabbit heart perfused with buffer containing human plasma.20,29 The cardiac damage could be modulated by exposure of the heart to sCR1. sCR1 has the capacity to intercept the catalytic subunit (C3b) of the C3 and C5 convertases of both the classic and alternative complement pathways.54 The specificity of sCR1 provides strong support for the concept that a direct cardiac cytotoxic action can be elicited via activation of the complement system.

Ecker and Gross showed that heparin inhibited sheep red blood cell lysis when the erythrocytes were exposed to guinea pig complement. On the basis of these studies, we designed an assay using rabbit plasma and washed human red blood cells. Our rationale was based on the concept that the presence of complement in rabbit plasma would lead to the lysis of human red blood cells.27 If our contention that heparin inhibits the cytolytic action of complement on red blood cells holds true, then the presence of heparin should attenuate the red blood cell lysis by rabbit plasma. The results of our studies using both heparin and N-acetyl heparin confirm this hypothesis. Further strength is added to our findings by the reverse experiment, in which heparin or N-acetyl heparin prevented the lysis of rabbit red blood cells by human plasma. Moreover, the selective inhibitor of complement activation, sCR1,54,55 prevented lysis of human red blood cells exposed to rabbit plasma.

The heparin molecule possesses independent anticoagulant and anticompartment sites21 and inhibits formation of the bimolecular complex C3bB by inhibiting the binding site for factor B on cell-bound C3b.53 The one mechanism shared by both heparin and N-acetyl heparin is the ability to inhibit activation of the complement system. The glycosaminoglycans, heparin and N-acetyl heparin protected the rabbit isolated heart against the cytotoxic effects of 4% human plasma added to the perfusion medium, an action possibly due to inhibition of the component cascade. The inability of r-hirudin, a specific thrombin inhibitor, to exert a protective effect under similar conditions provides compelling evidence that the protection afforded by heparin is not related to an antithrombin mechanism. The latter conclusion is consistent with the observation that the nonanticoagulant, N-acetyl heparin, also prevented the cytotoxic effect of human plasma on the isolated heart. The selective chemical modification of heparin, resulting in N-acetyl heparin, effectively reduces or negates the anticoagulant activity of the former compound without a concomitant loss in the ability to inhibit activation of the complement system.56

At present, the mechanism for the observed cardio-protection provided by the glycosaminoglycans is not known with certainty. Although it is intriguing to invoke a role for the contribution of tissue complement activation to the tissue injury associated with ischemia/reperfusion, the present study lacks direct evidence for this mechanism, despite the fact that inhibition of the complement cascade by glycosaminoglycans is documented in the literature. Whereas most plasma complement components are synthesized by hepatocytes, many components are synthesized by other cells, particularly mononuclear phagocytes, endothelial cells, fibroblasts,
and epithelial cells. It is entirely plausible, however, that the observed cardioprotection is unrelated to complement inhibition and may be the result of one or more of the aforementioned reported actions of the glycosaminoglycans. Continued investigation is warranted in an effort to gain deeper insight into the true mechanism of action.

The results of this investigation indicate that selective chemical modification of the heparin molecule will negate its antiocoagulant effects while retaining the potential to provide a cardioprotective action. The reported observation offers interesting opportunities concerning the potential clinical application of glycosaminoglycans as modulators of tissue injury.

Acknowledgments

This study was supported by grant HL-19782-15 from the National Institutes of Health, Heart, Lung, and Blood Institute. The authors thank Michael A. Schork, PhD, Professor of Biostatistics, University of Michigan School of Public Health, for consultation and review of the statistical methods used in this study.

References

42. Hiebert LM, McDuffie NM. The internalization and release of heparins by cultured endothelial cells: the process is cell specific, heparin source, time and concentration dependent. Artery. 1990; 17:107-118.
Effects of heparin and N-acetyl heparin on ischemia/reperfusion-induced alterations in myocardial function in the rabbit isolated heart.
G S Friedrichs, K S Kilgore, P J Manley, M R Gralinski and B R Lucchesi

_Circ Res._ 1994;75:701-710
doi: 10.1161/01.RES.75.4.701

_Circulation Research_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1994 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/75/4/701

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in _Circulation Research_ can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to _Circulation Research_ is online at:
http://circres.ahajournals.org//subscriptions/