Nonanticoagulant Heparin Prevents Coronary Endothelial Dysfunction After Brief Ischemia-Reperfusion Injury in the Dog

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Background—Coronary endothelial dysfunction after brief ischemia-reperfusion (IR) remains a clinical problem. We investigated the role of heparin and N-acetylheparin, a nonanticoagulant heparin derivative, in modulating coronary endothelial function after IR injury, with an emphasis on defining the role of the nitric oxide (NO)–cGMP pathway in the heparin-mediated effect.

Methods and Results—Male mongrel dogs were surgically instrumented, and the effects of both bovine heparin and N-acetylheparin on coronary endothelial vasomotor function, expressed as percent change from baseline flow after acetylcholine challenge, were studied after 15 minutes of regional ischemia of the left anterior descending artery (LAD) followed by 120 minutes of reperfusion. In dogs treated with placebo (saline), coronary vasomotor function was significantly ($P$ $\leq$ 0.03) decreased after 15 and 30 minutes of reperfusion (65±12% and 73±12%) compared with preischemia (103±6%). In contrast, the vasodilatory response to the endothelium-independent vasodilator sodium nitroprusside was maintained during reperfusion. Preischemic administration of both bovine heparin and N-acetylheparin (6.0 mg/kg IV) preserved coronary endothelial function throughout reperfusion. In a parallel group of dogs, nitrate/nitrite (NOx) and cGMP levels in the LAD were measured after treatment and during 15-minute reperfusion. Preischemic administration of N-acetylheparin caused a significant increase in basal NOx and cGMP levels compared with saline controls. Pretreatment with N-acetylheparin also caused a significant increase in NOx and cGMP levels in the LAD after 15 minutes of reperfusion compared with IR alone.

Conclusions—These results suggest that heparin preserves coronary endothelial function after brief IR injury by a mechanism independent of its anticoagulant activity and that the effect of heparin may be mediated in part by activation of the NO-cGMP pathway. (Circulation. 1999;99:1062-1068.)

Key Words: endothelium • endothelium-derived factors • heparin • ischemia • reperfusion

The endothelium regulates vascular function via the elaboration of vasoactive mediators that cause either vasodilation or vasoconstriction of the vascular smooth muscle. Since the discovery that an intact endothelium is required for agonist-stimulated vasodilation,1 endothelium-derived factors have been widely studied. The identification of endothelium-derived relaxing factor (EDRF) as nitric oxide (NO)2 and the physiological importance of NO has prompted an enormous amount of research in this area. At physiological concentrations, NO possesses a number of properties specific to the endothelium that make it ideal for the maintenance of vasomotor function during physiologic disturbances such as ischemia-reperfusion (IR) injury. NO is a potent vasodilator as well as an inhibitor of platelet aggregation, neutrophil activation, and adhesion to the endothelium.2,3 NO thus serves to maintain the homeostasis of the endothelium and prevent vasoconstriction, thrombosis, and neutrophil-mediated injury, which are all evident after IR injury.

Episodes of IR have been shown by several investigators to have deleterious effects on endothelial function.4,5 Endothelial dysfunction during IR is manifested in part by an impaired ability of the endothelium to elaborate NO both basally4 and after administration of endothelium-dependent agonists.4 Ma et al demonstrated that diminished endothelial elaboration of NO during reperfusion is associated with increased neutrophil adherence to the coronary endothelium,3 which contributes to the endothelial dysfunction observed during IR.6 Recent studies7,8 have also implicated a protective effect of NO in the setting of IR. Therefore, administration of

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pharmacological agents that maintain NO production in the setting of IR may preserve coronary endothelial function and prevent the adverse sequelae of reperfusion injury.

Heparin is one of the most widely used drugs in the clinical setting because of its anticoagulant activity. Heparin binds to antithrombin III and catalytically accelerates the inhibition of thrombin and factor Xa, resulting in anticoagulation. Heparin has also been shown to possess several properties in addition to its anticoagulant activity, including inhibition of the inflammatory response,9 scavenging of free radicals,10 and modulation of endothelial vasoactive mediators.11,12 Its ability to modulate several physiological processes may explain the recent observations that heparin preserves endothelial13–15 and myocardial16–18 function during disturbances such as IR injury. Recent studies14,16–18 have also demonstrated that the ability of heparin to modulate these processes may be independent of its anticoagulant properties. Given that the main side effect of heparin is bleeding, application of heparin derivatives completely devoid of anticoagulant activity is of clinical interest.

The purposes of the present study were (1) to establish that the protective effect of heparin on the coronary endothelium in the setting of brief IR injury is independent of its anticoagulant activity and (2) to elucidate the effect of nonanticoagulant heparin on the coronary endothelial NO-cGMP pathway as a possible mechanism of its protective properties during IR injury.

Methods

Surgical Preparation

Male mongrel dogs (n=44) weighing 25 to 30 kg were used for this study. All protocols were approved by the Animal Care and Use Committee of Georgetown University and conformed to the guiding principles of the American Physiological Society. Dogs were sedated with sodium thiopental (25 mg/kg) and were intubated, and anesthesia was maintained with halothane (1.5%). A left thoracotomy was performed through the fifth intercostal space, and the heart was instrumented (Figure 1). Animals were allowed ≥30 minutes to stabilize after instrumentation.

In Vivo Studies

Surgical preparation and collection of data were performed at an inspired oxygen fraction of 1.0 and a respiratory rate of 10 to 13 ventilations per minute. End-tidal halothane, carbon dioxide, and arterial oxygen saturation were continuously monitored. Body temperature was maintained in the normothermic range with a warming blanket. ECG, aortic pressure, left ventricular pressure, and left anterior descending coronary artery (LAD) flow were monitored continuously. Coagulation parameters including activated clotting, prothrombin, and partial thromboplastin times were measured before and 1 hour after treatment. All signals were electronically filtered, amplified, and digitally converted at a sampling rate of 200 Hz.

Coronary Ischemia and Reperfusion

After surgical instrumentation, dogs were randomly assigned to 1 of 3 treatment groups (n=18): saline, bovine heparin (Sigma Chemical Co), or the nonanticoagulant heparin derivative N-acetylheparin (Sigma). The dose of both heparin and N-acetylheparin was 6.0 mg/kg administered as a bolus intravenously 2 hours before our ischemic insult. The dose of heparin was determined on the basis of successful ischemia included cessation of LAD flow, wall-motion changes, cyanosis of the anterior wall, and typical ECG changes. To further prove that no significant collaterals were present, color-coded microspheres were injected before LAD occlusion and during 10 minutes of ischemia, and regional myocardial blood flow (RMBF) was measured as previously described.19

Coronary Vasomotor Function

We assessed coronary vasomotor function by infusing vasoactive drugs into the LAD and measuring the vasodilatory response. This value was expressed as percent change increase in LAD flow after infusion of the endothelium-dependent agonist acetylcholine (ACH; 1.0 µg/min) and the endothelium-independent agonist sodium nitroprusside (SNP; 10.0 µg/min). The doses of both drugs were based on experiments that yielded a submaximal vasodilatory response and resulted in reproducible increases without systemic hemodynamic changes.5

In Vitro Studies

Venous blood samples were drawn from the great cardiac vein before ischemia and during 15 minutes of reperfusion, the time point of maximal endothelial dysfunction in our model. Dogs (n=12) were treated with either saline or N-acetylheparin (6.0 mg/kg IV), and venous levels of nitrate/nitrite were measured. In a separate group (n=26), cGMP was measured in the LAD of dogs pretreated with saline or N-acetylheparin (6.0 mg/kg IV) in the absence or presence of 15-minute ischemia followed by 15-minute reperfusion.

Measurement of Nitrite/Nitrate and cGMP

Ion-exchange high-performance liquid chromatography was used to analyze nitrite/nitrate (NOx) levels as initially described by Romero et al.20 cGMP levels were measured in the LAD by standard radioimmunoassay as previously described.18
crease in LAD flow (20 mL/min) compared with baseline.

Administration of N-acetylheparin (6.0 mg/kg) protected the endothelium from IR injury, as evidenced by preservation of the endothelium-dependent vasomotor function (Figure 2). Bolus infusion of bovine heparin (16 mL/min) and subsequent administration of N-acetylheparin after L-NAME did not cause any measurable increase in LAD flow (20±4 mL/min).

Measurement of Nitrate and Nitrite
Administration of N-acetylheparin in the absence of IR caused a significant increase in basal NOx levels compared with saline control (Figure 5). In dogs pretreated with saline, NOx levels were significantly decreased during 15 minutes of reperfusion compared with preischemic values of NOx (Figure 5). Pretreatment with N-acetylheparin before the ischemic insult resulted in a significant increase in NOx levels during 15-minute reperfusion compared with IR alone (Figure 5). Treatment with N-acetylheparin during IR, however, did not restore NOx levels to the stimulated basal levels.

Measurement of cGMP Levels in LAD
In the absence of IR, administration of N-acetylheparin caused a significant increase in basal cGMP levels in the LAD compared with saline control (Figure 6). Pretreatment with N-acetylheparin in the setting of IR injury also caused a significant increase in cGMP compared with saline pretreatment (Figure 6). Treatment with N-acetylheparin failed to restore cGMP levels in the reperfused LAD to basally stimulated levels. Pretreatment with L-NAME significantly attenuated the increase in cGMP in groups treated with N-acetyl heparin alone and in the setting of IR injury.

Coagulation Parameters
Activated clotting, prothrombin, and partial thromboplastin times were all significantly elevated 1 hour after initiation of heparin treatment (Table 3). Administration of N-acetylheparin did not increase any of the coagulation parameters measured (Table 3), consistent with the inability

Statistical Analysis
Multivariate analysis was performed by ANOVA, with Scheffé test used to obtain levels of statistical significance for multiple comparisons. For analysis of in vivo results, repeated-measures ANOVA was used, with Dunnett test used to adjust for the multiple comparisons made with baseline values. A 2-tailed unpaired Student t test was used for comparison of values between groups. Values are expressed as mean±SEM. Statistical significance was achieved at a value of P<0.05.

Results
Hemodynamic Measurements and RMBF
Brief IR had no significant effect on heart rate, mean arterial pressure, left ventricular systolic pressure, or left ventricular end-diastolic pressure, all of which were comparable in the saline and N-acetylheparin-treated groups (Table 1). RMBF was significantly decreased during ischemia in all groups (Table 1).

Coronary Vasomotor Function
Baseline LAD flow remained stable throughout this experimental protocol (Table 2). In dogs pretreated with saline, endothelial function after ACh was significantly decreased during 15- and 30-minute reperfusion compared with preischemic values (Figures 2 and 3). Bolus infusion of bovine heparin (6.0 mg/kg) protected the endothelium from IR injury, as evidenced by preservation of the endothelium-dependent vasomotor function (Figure 2). Bolus infusion of N-acetylheparin (6.0 mg/kg IV) also preserved coronary endothelial function during early reperfusion (Figure 3). The vasodilatory response to SNP was maintained during a designated time point of reperfusion in all 3 groups and was not significantly affected by IR injury (Figure 4). In a separate series of experiments, administration of N^6-nitro-L-arginine methyl ester (L-NAME; 10 mg/kg) caused an increase in LAD flow (20±2 mL/min) compared with baseline
of this drug to bind to antithrombin III and produce anticoagulation.

Discussion

The results of the present study demonstrate that the preischemic administration of heparin preserves coronary endothelial function after brief IR injury by a mechanism independent of its anticoagulant properties and involving the NO-cGMP pathway.

The coronary endothelial dysfunction after IR injury observed in the present study is consistent with several previous studies that have demonstrated impaired coronary endothelium-dependent vasorelaxation in response to specific agonists. We have previously demonstrated that brief episodes of IR induce a temporary functional derangement in endothelium-dependent responses that does not cause any structural damage to the endothelium. This “endothelial stunning” is temporary and recovers during later periods of reperfusion, which is in contrast to other studies in which longer periods of ischemia result in structural and functional damage to the endothelium. The present findings confirm these observations that brief episodes of IR induce a temporary impairment of endothelium-dependent vasodilation during early reperfusion (15 and 30 minutes), which subsequently returns to normal after 60 and 120 minutes of reperfusion. Moreover, studies have demonstrated that impaired NO release in coronary arteries after IR injury occurs early during reperfusion.

The present findings are consistent with these observations given the diminished endothelium-dependent response as well as the attenuated basal levels of NOx and cGMP in the coronary circulation during 15-minute reperfusion.

Given the critical role that NO plays in the maintenance of normal homeostasis in the coronary endothelium, it is conceivable that pharmacological interventions that preserve NO would be beneficial. Several studies have demonstrated the salutary effect of interventions used to preserve physiological levels of NO during reperfusion injury. Cardioprotection and attenuation of coronary endothelial dysfunction has been demonstrated after administration of authentic NO, organic NO donors, and the NO precursor L-arginine. Furthermore, other pharmacological agents, including estradiol, growth factors, and bovine heparin, have been demonstrated to preserve agonist-stimulated NO activity from the coronary endothelium during reperfusion injury by a variety of mechanisms.

The present findings are in agreement with those of several investigators who have recently demonstrated that administration of heparin has a protective effect in the setting of IR injury. Heparin has been demonstrated to afford protection to both skeletal muscle and the myocardium after prolonged episodes of IR injury. In addition to preventing muscular necrosis, heparin has been shown to

Figure 2. Percent increase in LAD flow after challenge with ACh in dogs pretreated with saline (●) or bovine heparin (□). All animals (n=12) were subjected to 15-minute ischemia followed by 120-minute reperfusion. Data are expressed as mean±SEM. *P<0.05 vs preischemic saline-treated group.

Figure 3. Percent increase in LAD flow after challenge with ACh in dogs pretreated with saline (●) or the nonanticoagulant heparin derivative N-acetylheparin (■). All animals (n=12) were subjected to 15-minute ischemia followed by 120-minute reperfusion. Data are expressed as mean±SEM. *P<0.05 vs preischemic saline-treated group.
preserve endothelial function during reperfusion injury. In an isolated skeletal muscle model of IR injury, Sternbergh et al demonstrated that heparin and heparinoids prevented the endothelial dysfunction associated with reperfusion injury. The precise mechanism of how heparin and heparinoids afford protection to the coronary endothelium during IR injury has not been clearly addressed and studied to date. Therefore, the second aim of the present study was to focus on one possible mechanism of action of nonanticoagulant heparin and elucidate its effect on the NO-cGMP pathway in the coronary circulation.

Heparin is a complex linear glycosaminoglycan, endogenously produced in mast cells, that binds to antithrombin III at a unique pentasaccharide sequence essential for anticoagulation. Modification of this sequence on heparin by N-desulfation or N-acetylation results in heparin derivatives that are devoid of anticoagulant activity. One mechanism of action, presumably of both heparin and heparin derivatives, in the setting of endothelial IR injury is the avid association of heparin with the endothelium. The close relationship of heparin with the endothelium has been demonstrated in both cultured endothelial cells and in vivo models. Heparin binds to the luminal surface of the endothelial cell almost immediately after intravenous injection and becomes internalized by endocytosis. Heparin has recently been demonstrated to modulate the production and release of several endothelium-derived vasoactive mediators, including endothelin-1, nitric oxide (NO), and NO. Yokokawa et al and Piatti et al independently suggested that the heparin-mediated decrease in endothelin-1 production is NO-dependent. In a separate series of experiments, Yokokawa et al demonstrated that heparin promotes NO formation in cultured endothelial cells from spontaneously hypertensive rats as well as in humans heparinized for hemodialysis. NO levels in both of these studies were measured by the NOx metabolites, nitrate and nitrite, which is in accordance with the present results. In contrast, a recent study by Upchurch et al suggests that high doses of porcine heparin inhibit endothelial NO synthase (eNOS) activity and expression. The present study confirms reports that heparin increases NO production, because both NOx levels were increased in venous samples obtained from the great cardiac vein, and L-NAME–sensitive cGMP levels were also significantly elevated in N-acetylated–treated LAD during 15-minute reperfusion. An alternative pathway of cGMP activation, such as particulate guanylate cyclase, is unlikely given that L-NAME completely inhibited the heparin-mediated effect. A direct vasodilatory effect of heparin and N-acetylated heparin on the coronary endothelium cannot

**Figure 4.** Percent increase in LAD flow after challenge with SNP in dogs pretreated with saline (open bars), bovine heparin (solid bars), or N-acetylheparin (hatched bars). All animals (n=18) were subjected to 15-minute ischemia followed by 120 minutes of reperfusion. Data are expressed as mean±SEM.

**Figure 5.** NOx levels, measured as nitrate/nitrite, in the coronary circulation in dogs pretreated with saline or N-acetylheparin in the absence (open bars) or presence (solid bars) of IR. All animals (n=12) were subjected to 15 minutes of ischemia followed by 15 minutes of reperfusion. Data are expressed as mean±SEM. *P<0.05, N-acetylheparin vs saline treatment; +P<0.05, IR vs no IR injury.

**Figure 6.** cGMP levels in LAD in dogs pretreated with saline or N-acetylheparin in the absence (open bars) or presence (solid bars) of IR injury. All animals (n=26) were subjected to 15 minutes of ischemia followed by 15 minutes of reperfusion. Data are expressed as mean±SEM. *P<0.05, N-acetylheparin vs saline treatment; +P<0.05, IR vs no IR injury; #P<0.05, L-NAME vs saline and N-acetylheparin.

**TABLE 3. Coagulation Profile Before and After Treatment With Bovine Heparin and N-Acetylheparin**

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>Bovine Heparin</th>
<th>N-Acetylheparin</th>
<th>Normal</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACT, s</td>
<td>84.3±4.6</td>
<td>663.8±79*</td>
<td>71.7±11.4</td>
<td>60–90</td>
</tr>
<tr>
<td>PT, s</td>
<td>8.6±0.3</td>
<td>17.1±1.2*</td>
<td>8.8±0.3</td>
<td>6–12</td>
</tr>
<tr>
<td>PTT, s</td>
<td>12.6±0.8</td>
<td>&gt;100*</td>
<td>16.6±1.2</td>
<td>10–25</td>
</tr>
</tbody>
</table>

ACT indicates activated clotting time; PT, prothrombin time; and PTT, partial thromboplastin time. *P<0.05 compared with baseline values. Values are mean±SEM.
be concluded on the basis of the present findings. We have previously demonstrated, however, that both heparin and N-acetylheparin directly vasodilate bovine thoracic aorta in an NO-dependent manner.11

The present findings are the first to report that the mechanism of the heparin-mediated increase in NO activity in the coronary circulation is independent of the anticoagulant activity of heparin. One possible mechanism of the protective effect of nonanticoagulant heparin observed during IR injury may be secondary to the activation of coronary eNOS. We have recently demonstrated that heparin and nonanticoagulant heparin increase basal eNOS activity in cultured endothelial cells.35 Endothelial cells were exposed to heparin and N-acetylheparin for 2 hours, which is the same time course used in the present study. The pathway for eNOS activation, however, remains to be elucidated. After heparin is bound to the endothelium, it may alter specific second-messenger pathways that are critical for activation of eNOS. A recent study by Bezprozvanny et al13 demonstrates that heparin activates the ryanodine receptor on the sarcoplasmic reticulum, resulting in elevated levels of intracellular Ca2+, which may increase activation of eNOS. The role that neutrophils play in mediating the coronary endothelial dysfunction associated with IR injury has been well documented.6,34 NO donors22 and l-arginine23 have been demonstrated to preserve coronary endothelial function during IR injury. Therefore, the protective effect of nonanticoagulant heparin observed during IR injury may be secondary to NO-mediated inhibition of neutrophil accumulation on the coronary endothelium. Another possible mechanism may be a direct inhibition of the inflammatory response by nonanticoagulant heparin, given its ability to directly inhibit neutrophil accumulation and complement activation.9,16,17 Previous studies by Lucchesi et al16,17 have demonstrated that nonanticoagulant heparin inhibits complement activation and prevents myocardial dysfunction associated with reperfusion injury. Further studies are required to delineate the second-messenger pathways involved in heparin stimulation of NO production and its protective role during IR injury.

In conclusion, we report for the first time that heparin preserves coronary endothelial function in the setting of brief IR injury by a mechanism independent of its anticoagulant activity and that the protective mechanism is mediated in part by activation of the NO-cGMP pathway in the coronary circulation. The deleterious effect that IR injury has on coronary endothelial production of NO and the resultant sequelae of a dysfunctional endothelium may result in vasocnstriction, thrombosis, and reocclusion. Administration of a pharmacological therapy that preserves NO may serve to prevent endothelial injury during IR without the adverse complications of clinical bleeding.

Acknowledgments

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