High-Dose Heparin Impairs Nitric Oxide Pathway and Vasomotion in Rats

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Background—Platelet-activating effects have been reported with high-dose heparin in acute thrombotic disorders. Recent studies have shown that increased platelet aggregation is due to reduced nitric oxide (NO) production in endothelial cells cultured in the presence of high-dose heparin. The aim of this study was to determine whether heparin can affect the NO pathway and the regulation of the vascular tone in vivo.

Methods and Results—Anesthetized and mechanically ventilated Sprague-Dawley rats were treated with high-dose heparin. After 4 hours, the endothelial constitutive NO synthase (ecNOS) protein content in the aorta decreased (36% reduction, P<0.05), as detected by immunoblotting, and NO-dependent vascular reactivity was impaired. In fact, the increase in mean arterial blood pressure after inhibition of ecNOS with L\textsuperscript{-}nitro-arginine methyl ester (30 mg/kg) was smaller in heparin-treated animals than in controls (+26.9±4.8 versus +48.3±9.1 mm Hg, P<0.05), and further infusion of the biological ecNOS substrate L-arginine (0.5 g/kg) was ineffective in reversing systemic vasoconstriction (−1% versus 28% vasodilatation, P<0.001).

Conclusions—High-dose heparin can significantly affect vascular reactivity in vivo by downregulation of ecNOS protein expression. (Circulation. 1999;99:2861-2863.)

Key Words: heparin ■ nitric oxide ■ endothelium

Heparin is the most widely used anticoagulant in current clinical practice. However, there is growing evidence that heparin can induce platelet activation. Recent studies report that therapeutic doses of heparin increase spontaneous and stimulated platelet aggregation when administered during acute coronary syndromes or unstable angina, but also in healthy subjects.\textsuperscript{1–3} This effect is associated with impaired endothelial nitric oxide (NO) synthesis in bovine aortic endothelial cells exposed to high-dose heparin.\textsuperscript{4}

Endothelial synthesis of NO plays a fundamental role both in the regulation of platelet reactivity and in the control of vascular tone.\textsuperscript{5} However, the effects of high-dose heparin on NO-mediated vasoreactivity in vivo have never been addressed. This effect would be clinically relevant in procedures such as PTCA and cardiopulmonary bypass, in which an activated partial thromboplastin time by 2 to 2.5 times (n=6) and >4 times (n=8) control values, respectively, at 4 hours. Control rats (n=7) received an intravenous bolus of saline. Blood pressure was recorded with a transducer (Statham P23 XL) and the signal displayed on a polygraph (Gould RS 3400). We assessed NO-dependent vascular tone by infusing 30 mg/kg L-NAME (Sigma), a competitive inhibitor of NOS.\textsuperscript{6} After 10 minutes, 0.5 g/kg L-arginine hydrochloride (Sigma) was administered to restore ecNOS activity. Mean arterial blood pressure (MAP) was monitored throughout the experiments.

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The animals were killed, and the thoracic and abdominal aorta was quickly removed and placed in ice-cold saline solution. The adventitial tissue was cleaned off with care to avoid damage to the endothelium. The specimens were then frozen in liquid nitrogen.

**ecNOS Immunoblotting**

ecNOS immunoblotting was performed in aortic extracts as previously described. Mouse monoclonal anti-human ecNOS (Affiniti) and peroxidase-conjugated rabbit anti-mouse IgG (Dako) were used as primary and secondary antibodies, respectively. The specific signal was detected with an enhanced chemiluminescence system (ECL, Amersham) and quantified by densitometry. Each sample was processed 3 times.

**Statistical Analysis**

Results are expressed as mean±SEM. Data were analyzed by 2-way ANOVA for repeated measures followed by post hoc tests and by Student’s t test. A value of *P*<0.05 was considered significant.

**Results**

**Effect of Heparin on Regulation of Vascular Tone**

Heparin did not affect baseline MAP 4 hours after injection compared with control animals (*P*=0.2). After administration of L-NAME 30 mg/kg, MAP increased markedly in the control group (+48.3±9.1 mm Hg, *P*<0.005) (Figure 1). Infusion of L-arginine 0.5 g/kg restored MAP to baseline values (from 145.3±13.3 to 105.3±13.7 mm Hg, *P*<0.005). d-Arginine had no effect (data not shown). Low-dose heparin did not affect vasoconstriction induced by L-NAME but reduced vasodilatation after L-arginine (from 127.2±8.2 to 116.0±10.3 mm Hg, *P*=0.07) (Figure 1). The increase in MAP after L-NAME was blunted in rats treated with high-dose heparin (+26.9±4.8 mm Hg, *P*<0.05 versus controls by post hoc test), and vasodilatation by L-arginine was abolished (from 103.1±12.5 to 104.2±13.8 mm Hg, *P*=0.7) (Figure 1).

To assess whether the observed impairment of vascular reactivity was influenced by L-NAME/L-arginine sequestration by heparin, MAP was measured after 30 minutes of high-dose heparin. At this time point, the changes in MAP caused by L-NAME/L-arginine were similar to controls and different from those observed at 4 hours (*n*=4, *P*<0.01 at the L-NAME level).

**Effect of Heparin on ecNOS Protein Expression**

Low-dose heparin at 4 hours had no effect on ecNOS expression (17.64±1.38 versus 16.90±1.51 mU OD/µg protein in controls) in the rat aorta, as determined by densitometric analysis after ecNOS immunoblotting (Figure 2). High-dose heparin at 4 hours significantly decreased ecNOS protein expression (10.76±1.13, *P*<0.05 versus control and low-dose heparin) (Figure 2). No effect of high-dose heparin on ecNOS expression was observed at 30 minutes (17.93±1.51 mU OD/µg protein, *n*=4, *P*<0.01 versus 4 hours of high-dose heparin). Only a negligible amount of ecNOS was immunoblotted in endothelium-denuded vessels, which suggests that the observed changes of ecNOS protein occurred in the endothelium.

**Discussion**

The results indicate that high-dose heparin in vivo downregulates ecNOS protein expression, thus affecting the normal vascular reactivity.

A decrease in NO production associated with reduced ecNOS mRNA and protein expression has been reported by Upchurch et al after prolonged (4 hours) high-dose heparin treatment of endothelial cells. In the same study, this effect was correlated with increased platelet aggregation. This effect was also observed in patients with unstable angina after therapeutic doses of unfractionated heparin.

Conversely, other studies have shown that heparin favors the release of NO in cultured vascular endothelial cells of rats, pigs, and humans. It has also been shown that heparin causes NO-mediated vasodilatation of isolated coronary arterioles in pigs and prevents coronary endothelial dysfunction associated with ischemia-reperfusion injury in dogs. The increase in NO-mediated vasodilatation by heparin may be indirect and due to the reaction between heparin and xanthine oxidase, which decreases superoxide formation.

Another possible explanation for these discrepancies may be related to the variable time of exposure to heparin in the different protocols. Studies reporting a stimulating effect on NO production also used high concentrations of heparin, but for a period of time not exceeding 1 hour. In our study, heparin exposure was set at 4 hours so as to mimic the condition of continuous heparin administration clinically applied in acute cardiovascular procedures. It may be hypothesized that heparin given as short-term treatment acts as an ecNOS agonist, but the effects on gene transcription at these high doses may occur only with prolonged stimulation. It has been reported that heparin influences gene expression, ie, by...
inhibiting the expression of the proto-oncogene c-fos as well as that of endothelin-1, collagenase, and tissue plasminogen activator genes.14–16 Although we did not assess ecNOS mRNA, the clear decrease in ecNOS protein expression induced after high-dose heparin after 4 hours but not after 30 minutes of infusion is likely to be due to reduced ecNOS gene transcription.

Our data showed reduced ecNOS protein expression accompanied by an impaired hemodynamic response after infusion of the ecNOS antagonist L-NAME, suggesting reduced baseline NO production. Interestingly, in the heparin-treated animals, administration of L-arginine did not restore the pre–L-NAME values of arterial pressure. The lack of effect of L-arginine in lowering blood pressure suggests that the downregulated ecNOS enzyme is also impaired by high-dose heparin. A degree of impairment was in fact seen with low-dose heparin, although downregulation of ecNOS was observed only at high doses. Such high doses of heparin are routinely used in PTCA and cardiopulmonary bypass and may well be of clinical relevance.

The bioavailability of L-NAME/L-arginine may also be reduced by a direct reaction between the polyanionic heparin and the cationic guanidino side chain of the ecNOS blocker/substrate. This interaction is relevant in vitro: we found a 27% reduction of L-[3H]arginine transport through the plasma membrane of human umbilical vein endothelial cells when 5 U/mL heparin was added to the uptake medium (data not published). However, 30 minutes of heparin infusion did not affect the NO-dependent modulation of vasoreactivity, arguing against a rate-limiting role played by ionic forces in vivo.

In conclusion, our experiments suggest that high-dose heparin, given as a prolonged (4 hours) specific therapeutic regimen, can negatively influence the NO pathway, impairing the endothelium-dependent regulation of vascular tone.

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