Heparin Interferes with the Biological Effectiveness of Atriopeptin

YUEFANG WEI, SANDRA W. HOLMBERG, KATHLEEN M. LEAHY, PETER O. OLINS, CATHERINE S. DEVINE, AND PHILIP NEEDLEMAN

SUMMARY The chromatographic mobility of atriopeptin-28 or of the prohormone is markedly altered by preincubation of the peptides with heparin before separation on reverse-phase high performance liquid chromatography. Protamine prevented the heparin effect and reestablished the original migration pattern of the atrial peptides. The addition of heparin to either rat or human plasma samples did not interfere with the atriopeptin immunoreactivity. The influence of heparin on the biological activity of the atriopeptin-28 in anesthetized rats was also investigated. Infusion of heparin (30 U/min) significantly reduced the dose-dependent fall of blood pressure produced by atriopeptin-28, but did not interfere with the hypotensive effect of nitroglycerin. Similarly, infusion of heparin in volume-expanded rats markedly decreased the diuresis produced by atriopeptin-28 without altering the urine volume excreted in response to furosemide. These data suggest that the highly charged molecule heparin can modify the physical and biological properties of atriopeptins, perhaps by binding to the numerous arginine residues (i.e., 5 arginine residues in atriopeptin-28) in the atriopeptin molecules (Hypertension 9: 607-610, 1987)

KEY WORDS • atriopeptin • heparin • blood pressure • diuresis • atrial natriuretic factor • atrial peptide

ATRIOPEPTIN (AP) is stored as a 126-residue prohormone (AP126) in secretory granules of mammalian atrial cardiocytes. The circulating form of the peptide is AP28, which exerts numerous biological effects, including diuresis, natriuresis, vasodilation, and reversal of hypertension. The processing of prohormone (AP126) to the C-terminal hormone (AP28) occurs rapidly in the myocardium at the time of release, although the site and enzymatic mechanism of this distinct proteolytic cleavage are unresolved. The cleavage of the prohormone can be produced in vitro by incubation with trypsin, kalikrein, platelets, and thrombin. Thrombin cleaves the prohormone after the Pro-Arg residues to liberate the C-terminal AP28.

We used heparin to investigate the possible effect of endogenous thrombin on the cleavage of atriopeptigen (AP126). Interestingly, we found that the retention time of AP28 on reverse-phase high performance liquid chromatography (HPLC) was markedly changed in plasma extract from the heparin-treated rats. This finding suggested the possibility that polyanionic heparin could readily complex with AP28, which contains five arginine residues. Such a charged complex could result in altered physical and biological properties. In this study we examined the influence of heparin on the chromatographic mobility and the diuretic-vasodilator activities of AP.

Materials and Methods

HPLC and Radioimmunoassay

Synthetic rat AP28 and a recombinant prohormone AP116 (AP114-Arg-Arg; i.e., residues 13-128 of the native prohormone expressed by the atrial DNA) were incubated with heparin or furosemide on ice for 5 minutes and then applied to reverse-phase HPLC on a Vydac C18 column. The following linear gradient was used at the rate of 1 ml/min: 15 to 40% A in 30 minutes; 40 to 55% by 60 minutes A = 0.05% trifluoracetic acid and B = 100% acetonitrile/0.05% trifluoroacetic acid). The fractions were lyophilized and reconstituted in 100 mM phosphate buffer (pH 7.4) for radioimmunoassay. To determine the effect of heparin on the peptides, protamine
sulfate (Eli Lilly & Co., Indianapolis, IN, USA) was added to the incubation of heparin and APs, followed by centrifugation at 3000 g to remove the precipitate prior to HPLC separation.

Radioimmunoassay was performed as previously described. \(^{10}\) Briefly, the fractions were incubated with 10,000 cpm per tube \(^{125}\)I-AP24, and a 1:300,000 final dilution of guinea pig antiserum (GP-4) was generated against synthetic rat AP24. Antibody-bound radioligand was incubated overnight at 4°C using a 1:10,000 final dilution of goat anti-guinea pig IgG antiserum (Linco Research, Eureka, MO, USA). After centrifugation at 3000 g for 30 minutes, the pellet was washed with 0.25% bovine serum albumin/10 mM NaN\(_3\) and radioactivity was counted in a Micromedic gamma counter (ICN Biomedicals, Costa Mesa, CA, USA).

Bioassay
Female Sprague-Dawley rats (Sasco, Omaha, NE, USA) weighing 240 to 265 g were anesthetized with sodium pentobarbital (30 mg/kg i.p.). A femoral artery was cannulated, and the blood pressure was monitored with a Beckman R511 physiograph (Palo Alto, CA, USA) connected to a type 9853 H pressure transducer. In the blood pressure bioassay, one femoral vein was used for infusion of 0.225% sodium chloride/5% dextrose, with or without heparin (Sigma, St. Louis, MO, USA), at the rate of 0.01 ml/min for 30 minutes. The opposite femoral vein was cannulated for bolus injection of AP28 during the heparin infusion. Nitroglycerin (gyceryl trinitrate; Eli Lilly) was used as a control. In the diuresis bioassay, dextrose-saline with or without heparin was infused into one femoral vein at 0.15 ml/min for 60 minutes to induce volume expansion. The opposite femoral vein was cannulated for infusion of AP28 (0.01 ml/min for 30 minutes) during heparin infusion. The bladder was catheterized for urine collection. Furosemide was used as a control.

Results
Effects of Heparin on Migration and Immunoreactivity of Atriopeptins
On reverse-phase HPLC, the recombinant prohormone AP116 has a retention time of 34 minutes, as determined by both ultraviolet absorbance and immunoreactivity. After incubation of the prohormone with 25 units of heparin prior to HPLC, the peak of immunoreactivity was detected at 4 minutes. When protamine (0.5 mg) was preincubated with heparin and then added to AP116, subsequent HPLC analysis demonstrated that ultraviolet absorbance and immunoreactivity exhibited the same column retention time (i.e., 34 minutes) as the standard AP116 alone. The same results were observed in the experiment with AP28: the standard peptide alone or the peptide plus precomplexed heparin and protamine exhibited a 23-minute retention time, while AP28 plus heparin eluted at 4 minutes.

To determine whether the effect of heparin was due to interference in the radioimmunoassay, a number of in vitro control experiments were performed. Addition of heparin (up to 500 U/ml) directly to AP in the immunoassay tube did not shift the standard curve. However, the addition of protamine (0.5 mg) in the presence or absence of heparin depressed the standard AP binding curve. The addition of a wide range of heparin concentrations to plasma samples containing very low or very high levels of endogenous or exogenous AP did not alter the quantitative immunoreactivity (Table 1).

Effects of Heparin on Atriopeptin 28-Induced Hypotension and Diuresis
Bolus injections of AP28 into pentobarbital-anesthetized rats induced a dose-dependent decrease of systemic blood pressure (Figure 1). After infusion of heparin (30 U/min for 30 minutes), the hypotensive effect of AP28 was reduced significantly \((p<0.01)\). Nitroglycerin, a direct-acting vasodilator, caused a dose-dependent fall of blood pressure that was unaltered by heparin administration (see Figure 1, right side).

A similar phenomenon was observed in diuresis experiments. AP28 infusion in volume-expanded rats produced a dose-dependent diuresis (Figure 2). Administration of heparin (10 and 30 U/min) produced a marked decrease \((p<0.005\) and 0.001, respectively) of the diuresis caused by infusion of AP28 (300 ng/min). Furosemide (4 mg/kg bolus i.v.) increased urine volume threefold to fourfold; thus, the pretreatment control urine flow was 44 ± 5 µl/min and increased with furosemide to 106 ± 16 µl/min \((n = 8)\). When the diuresis experiment was repeated in the presence of an infusion of heparin (30 U/min), the pretreatment control urine volume was 66 ± 12 µl/min and rose to 136 ± 11 µl/min \((n = 6)\) in response to furosemide, thereby indicating no heparin-induced alteration in the sensitivity to the diuretic.

Discussion
We have demonstrated that heparin markedly decreased AP28-induced hypotension and diuresis in rats and altered the chromatographic mobility of AP28 and prohormone AP116. Trippodo et al. \(^{8}\) reported that in vitro incubation of rat atrial high molecular weight peptide (HMWP) with rat fresh blood or platelets, which contain heparin, inhibited the conversion of HMWP to low molecular weight peptide (LMWP) resulting in decreased natriuretic activity. They also used the histamine-contracted rabbit aorta to examine the effect of heparin on the conversion by incubation of HMWP with rat platelets and found that heparin inhibited the conversion of HMWP to LMWP and significantly reduced the vasorelaxation of HMWP. In view of the current experiments, platelet processing of the prohormone might have been reduced because the peptide-heparin complex may limit access to the proteolytic enzyme. Heparin is a heterogeneous group of
TABLE 1. Effect of Exogenous Heparin on the Atriopeptin Immunoreactivity of Rat or Human Plasma Samples

<table>
<thead>
<tr>
<th>Plasma samples</th>
<th>Heparin addition (U/ml)</th>
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<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Atriopeptin immunoreactivity (ng/ml plasma)</td>
<td></td>
</tr>
<tr>
<td>Rat plasma + exogenous AP28</td>
<td>15.9</td>
</tr>
<tr>
<td>Plasma from dAVP-treated rats</td>
<td>23.8</td>
</tr>
<tr>
<td>Atriopeptin immunoreactivity (pg/ml plasma)</td>
<td></td>
</tr>
<tr>
<td>Normal human plasma</td>
<td>55</td>
</tr>
<tr>
<td>Cardiomyopathic human plasma</td>
<td>654</td>
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Blood was collected from a chloral hydrate (350 mg/kg i.p.)-anesthetized rat in 50 mM EDTA (9:1 [vol/vol] blood/EDTA), centrifuged at 10,000 g for 1 minute, and the rat 28-amino acid peptide atriopeptin-28 (AP28; 15 ng/ml) was added. The heparin was preincubated with the plasma on ice for 30 minutes before immunoassay. Desamino-arginine vasopressin (dAVP) was administered (10 μg/kg i.v.) to chloral hydrate-anesthetized rats, and blood was collected after 5 minutes into 50 mM EDTA (9:1), centrifuged, and preincubated with heparin (as described above) before immunoassay.

The human plasma samples were collected in EDTA-coated Vacutainers and were preincubated with plasma before assay.

**Figure 1.** Effect of heparin on the systemic vasodilation produced by atriopeptin-28 (AP-28) and nitroglycerin (glyceryl trinitrate, GTN). Rats were infused with 0.225% NaCl/5% dextrose at 0.01 ml/min containing no heparin or 30 U/min heparin for 30 minutes. Agonists were injected intravenously. Values represent the mean ± SEM for four animals from each group.

**Figure 2.** Effect of heparin on the changes in urine volume induced by atriopeptin-28 (AP-28) in volume-expanded rats. Anesthetized rats were infused with 0.225% NaCl/5% dextrose at 0.15 ml/min for 60 minutes to induce a volume expansion. In the experimental group, heparin was added at the infusion doses of 10 and 30 U/min, respectively. Values represent the mean ± SEM for six animals from each group. Double (p<0.005) and triple asterisks (p<0.001) indicate significant difference compared with control value.

References
4. Currie MG, Geller DM, Cole BR, et al. Bioactive cardiac straight-chain anionic mucopolysaccharides. There are 10 and five arginine residues in AP16 or HMWP and AP28 molecules, respectively, which are positively charged at neutral pH. Therefore, heparin could bind to the arginines in the AP molecules, which would cause the complexed peptide to exhibit markedly decreased vasorelaxant activity and suppressed natriuresis and diuresis. Protamine sulfate is strongly basic because of its high arginine content. In vitro, protamine sulfate combines with heparin to form a stable complex and prevents the ability of heparin to interfere with the AP effect.

The plasma level of AP28 in normal subjects is approximately 40 pg/ml. Our results indicate that 25 U of heparin changed the physical properties of 2 μg of AP28 and 10 μg of AP16, suggesting that a heparin dose of 15 U/ml (a common anticoagulant concentration) may interfere with the measurement of APs. Storing plasma samples with heparin has been reported to decrease AP immunoreactivity by 20% relative to the samples collected in EDTA. Clinically, an intravenous injection of a 10,000-U bolus of heparin is not uncommon. The results from our experiments demonstrated that a 900-U dose of infused heparin decreased the fall of blood pressure induced by 10 μg of AP28, and infusion of 600 to 1800 U of heparin reduced diuresis produced by 2.5 μg of AP28 (300 ng/min/kg for 30 minutes). These data suggest that the dose of heparin used clinically may interfere with biological activities (but not the immunoreactivity) of endogenous AP by forming an AP-heparin complex.
substances: potent vasorelaxant activity in mammalian atria.
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