The Effects of Low Molecular Weight and Standard Heparin on Calcium Loss From Fetal Rat Calvaria

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Osteoporosis is a well-recognized complication of long-term heparin use. However, the mechanisms by which heparin can influence bone metabolism are unclear. We report here that unfractionated heparin stimulates the process of bone resorption and that the low molecular weight heparins (LMWHs), enoxaparin, fragmin, logiparin, and ardeparin produce significantly less calcium loss than unfractionated heparin. To assess calcium loss from bone, we quantified the release of $^{45}$Ca into the culture medium of fetal rat calvaria. $^{45}$Ca release was increased in a dose-dependent manner by the addition of either unfractionated heparin or the LMWHs; but more than 50-fold higher LMWH concentrations were required to obtain an equivalent effect to unfractionated heparin. Thus, at concentrations $\geq 2$ mg/mL (0.35 anti-Xa units/mL), unfractionated heparin stimulated $^{45}$Ca release 1.53 $\pm$ 0.06-fold. $^{45}$Ca release was increased to a similar extent by the addition of either $10^{-7}$ mol/L parathyroid hormone (PTH) or $10^{-6}$ mol/L 1.25 dihydroxyvitamin $D_3$ ($1.25 \text{ Vit D}_3$). In contrast to unfractionated heparin, LMWH concentrations $\geq 100$ mg/mL (>14.0 anti-Xa units/mL) were required before maximum isotope release was observed. At concentrations well above therapeutic levels, the LMWHs stimulated $^{45}$Ca release by only 1.25 $\pm$ 0.01-fold. Heparins with high and low antithrombin III affinities stimulated $^{45}$Ca release equally well. Both size and sulfation were found to be major determinants of heparin’s ability to promote isotope release. Thus, the ability of defined heparin fragments to stimulate $^{45}$Ca release correlated with their molecular weight, and after N-desulfation the ability of heparin to induce isotope release was greatly diminished. Dermatan sulfate had no effect on $^{45}$Ca release. We conclude that size and sulfation are major determinants of heparin’s ability to promote bone resorption and that the risk of heparin-induced osteoporosis may be reduced by the use of LMWH preparations.

MATERIALS AND METHODS

Materials. BGJb medium (Fitzton-Jackson modification) and pencillin/streptomycin solution were obtained from GIBCO BRL, Inc (Burlington, Ontario). Calcium-45 and 1.25 dihydroxyvitamin $D_3$ were obtained from ICN Biomedicals, Inc (Irvine, CA). Human synthetic parathyroid hormone (PTH) and N-desulfated heparin was purchased from Sigma Chemical Co (St Louis, MO). Heparin fragments of defined molecular weight were purchased from Enzyme Research Labs (South Bend, IN). Unfractionated heparin (175 U/mg) and enoxaparin (154 U/mg) were a generous gift from Rhone-Poulenc Rorer (Montreal, Canada). Fragmin (168 U/mg) was a generous gift from Kabi Pharmacia (Stockholm, Sweden), ardeparin (119 U/mg) a gift from Wyeth-Ayerst (Philadelphia, PA), and logiparin (106 U/mg) a gift from Novo Nordisk (Copenhagen, Denmark).

Calvaria isolation and culture. Pregnant Sprague-Dawley rats (Charles River Laboratories; St Constant, Quebec) were injected subcutaneously with 40 $\mu$Ci of $^{36}$Ca on their 18th day of gestation. Forty-eight hours later, the calvaria of the fetal rats were isolated under sterile conditions, and each parietal bone placed into a siliconized (1% AquaSil; Pierce, Rockford, IL) culture well containing 1.0 mL BGJb medium and 1% antibiotic/antimycotic solution. The bones were then cultured for 4 days at 37°C in the presence or absence of unfractionated heparin or one of the commercially available LMWHs. Alternatively, in some experiments the calvaria were exposed to ultraviolet (UV) irradiation for 24 hours to kill existing bone cells, before incubation with unfractionated heparin. On day 4, the culture supernatants were collected and the bone digested overnight in 10% trichloroacetic acid (TCA). The release of $^{36}$Ca into the assay medium was calculated as a percentage of the total radioactivity in each parietal bone and is expressed as a T/C ratio where:

$$\text{T/C Ratio} = \frac{\% \text{ of } ^{36}\text{Ca Released From Treated Calvaria}}{\% \text{ of } ^{36}\text{Ca Released From Control (Medium Only) Calvaria}}$$

Preparation of chemically modified low-affinity heparin (LAH). LAH was prepared from unfractionated porcine mucosal heparin (178 U.S.P units/mg; Sigma Chemical Co) according to the method of Casu et al. Briefly, unfractionated heparin (500 mg) was oxidized by sodium periodate in an aqueous medium for 24 hours at 4°C. The reaction was stopped by addition of ethylene glycol, dialyzed...
agains distilled water, and the oxidized heparin reduced by the addition of sodium borohydride. The solution was then adjusted to pH 3.0 to destroy excess borohydride and quickly readjusted to pH 7.0 to minimize acidic conditions. After a second overnight dialysis against distilled water, the solution was adjusted to 1.0 mol/L NaCl with solid NaCl salt and the LAH precipitated with three volumes of absolute ethanol. The product was recovered by centrifugation (2,000 g for 90 minutes at 4°C) and air-dried. Remaining traces of high-affinity material was removed by affinity chromatography on an antithrombin III-Sepharose column. This LAH fraction contains <1.0 U/mg of antifactor Xa activity.

Preparation of high- and low-affinity heparin by affinity chromatography. High-affinity heparin was obtained from porcine mucosal heparin (178 USP units/mg) by affinity chromatography on immobilized antithrombin III. Briefly, 50 mg of antithrombin III was reacted at pH 8.5 with 5 mL of CNBr-activated Sepharose 4B, and the resulting gel adsorbent equilibrated in a column with 0.1 mol/L Tris-HCl, pH 7.4, containing 0.2 mol/L NaCl. Equilibration buffer containing unfraccionated heparin was then applied to the column and the unadsorbed LAH collected in the effluent. Bound high-affinity heparin was eluted in 0.1 mol/L Tris-HCl, pH 7.4, containing 2 mol/L NaCl. Appropriate fractions were pooled, and recovered by precipitation with three volumes of absolute ethanol, as described above, before determining their effect on bone.

Statistical analysis. Statistical differences between the experimental and control groups in the calvaria experiments were determined by analysis of variance. If a significant difference between experimental and control groups was detected, an unpaired Student's t-test was performed at each point. Significance levels were adjusted as described above, before determining their effect on bone.

RESULTS

Stimulation of calcium release by PTH and 1,25 dihydroxyvitamin D3. The release of ⁴⁵Ca into the culture medium of fetal rat calvaria was promoted in the presence of either PTH (Fig 1A) or 1,25 dihydroxyvitamin D3 (1,25 vit D₃, Fig 1B). Both agonists stimulated ⁴⁵Ca release in a dose-dependent manner. Maximum ⁴⁵Ca release was observed in the presence of either 10⁻⁷ mol/L PTH or 10⁻⁸ mol/L 1,25 vit D₃. At optimal PTH or 1,25 vit D₃ concentrations, ⁴⁵Ca release was stimulated 1.75 ± 0.13 and 1.55 ± 0.06-fold, respectively (P < 0.001). In the absence of either agonist, calvaria cultured in the presence of media alone released 16.1% ± 0.3% of their total ⁴⁵Ca (mean of 18 experiments).

Effect of unfractionated heparin on ⁴⁵Ca release from fetal rat calvaria. Figure 2 demonstrates that calvaria cultured in the presence of unfractionated heparin released significantly more ⁴⁵Ca than did those cultured in the presence of media alone. The effect of unfractionated heparin was dose-dependent with maximal isotope release occurring at heparin concentrations ≥ 2 μg/mL (Fig 2). At therapeutic concentrations of 0.35 U/mL, unfractionated heparin stimulated isotope release 1.53 ± 0.06-fold (mean of 5 experiments; P < 0.001). The effect of heparin on ⁴⁵Ca release was cell-mediated as heparin was unable to promote ⁴⁵Ca release when cocultured with nonviable, UV irradiated calvaria (data not shown).

In the above experiments, ⁴⁵Ca release was measured after a 4-day coincubation of unfractionated heparin with prelabeled calvaria. Figure 3 demonstrates that although the majority of ⁴⁵Ca was released within the first 24 hours, isotope release increased linearly with time. After 4 days, the release of ⁴⁵Ca, promoted by unfractionated heparin, was maximal. No significant difference was found between unfractionated heparin and PTH in terms of their ability to induce ⁴⁵Ca release (Fig 3). In contrast to unfractionated heparin, the LMWH enoxaparin (at concentrations ≤ 10 μg/mL) was unable to promote the release of ⁴⁵Ca (Fig 3).

Effect of LMWH on ⁴⁵Ca release from fetal rat calvaria. To further define the ability of LMWHs to induce ⁴⁵Ca release, prelabeled fetal rat calvaria were cocultured with sev-
eral different commercially available LMWHs. Figure 4A through D demonstrates that \( ^{45}\text{Ca} \) release was promoted in a dose-dependent manner by the addition of the LMWHs, enoxaparin, logiparin, fragmin, and ardeparin. In contrast to unfractionated heparin, LMWH concentrations \( \geq 10 \mu g/mL \) required before maximum isotope release was observed (Fig 2 v Fig 4A through D). No significant difference was found between the LMWHs in terms of their ability to promote \( ^{45}\text{Ca} \) release. At optimal concentrations, the LMWHs stimulated isotope release 1.25-\( \pm 0.01 \)-fold \( (P < .001) \) compared with 1.53-\( \pm 0.06 \)-fold for unfractionated heparin.

**Effect of anticoagulant versus nonanticoagulant heparin on \( ^{45}\text{Ca} \) release.** To determine if the antithrombin III binding site on heparin was important or if the effect of heparin on bone was independent of its anticoagulant activity, we determined the ability of LAH to promote \( ^{45}\text{Ca} \) release. Heparin was fractionated by affinity chromatography on an anti-thrombin III column, and the high- and low-affinity fractions were tested for their ability to promote \( ^{45}\text{Ca} \) release. A second LAH was prepared by oxidation of unfractionated heparin with sodium periodate because fractionation by affinity chromatography can result in high and low affinity heparins with differing mean molecular weights.\(^{30}\) Figure 5 demonstrates that low- and high-affinity heparins stimulated \( ^{45}\text{Ca} \) release equally well, regardless of the method of preparation.

**Effects of molecular weight and degree of sulfation on \( ^{45}\text{Ca} \) release.** To determine if molecular size was a major determinant of heparin-induced \( ^{45}\text{Ca} \) release, we cocultured prelabeled fetal rat calvaria with heparin fragments of defined molecular weight. Fragments with a mean molecular weight \( \leq 5 \text{kD} \) had no effect on \( ^{45}\text{Ca} \) release (Fig 6). In contrast, both a 9-kD and an 18-kD fragment stimulated the release of \( ^{45}\text{Ca} \). The ability of a given fragment to promote \( ^{45}\text{Ca} \) release correlated directly with its mean molecular weight (Fig 6).

The degree to which sulfation affected the ability of heparin to induce \( ^{45}\text{Ca} \) release was also examined. Table 1 demonstrates that heparin’s ability to stimulate \( ^{45}\text{Ca} \) release was greatly diminished following N-desulfation. At a concentration of 10 \( \mu g/mL \), the effect of unfractionated heparin on \( ^{45}\text{Ca} \) release was maximal, while N-desulfated heparin was without effect. At 100 \( \mu g/mL \), N-desulfated heparin promoted only half as much \( ^{45}\text{Ca} \) release as did unfractionated heparin. Similarly, the less sulfated glycosaminoglycan, dermatan sulfate, was unable to promote \( ^{45}\text{Ca} \) release at any concentration (Table 1). At concentrations as high as 500 \( \mu g/mL \), calvaria cultured in the presence of dermatan sulfate released the same amount of \( ^{45}\text{Ca} \) as those cultured in the presence of media alone (data not shown).

**DISCUSSION**

We have adapted a reproducible experimental model to quantify heparin-induced calcium loss from bone. Thus, using a well-described rat calvaria model to measure bone resorption, we have obtained convincing evidence that the
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Fig 4. A comparison of the commercially available LMWHs. Prelabelled fetal rat calvaria were coin-cubated for 4 days at 37°C with increasing concentrations of either (A) enoxaparin, (B) logiparin, (C) fragmin, or (D) ardeparin. 4Ca release into the assay medium was calculated as a percentage of the total radioactivity in each parietal bone and is expressed as a T/C ratio (mean ± SEM). *P < .005 when compared with the release of isotope from calvaria cultured in the presence of media alone.

Commercially available LMWHs produce significantly less calcium loss than unfractionated heparin. We have also determined the structural determinants on heparin responsible for this loss. Thus, both size and degree of sulfation were found to be major determinants of heparin's ability to effect bone resorption.

The pathogenesis of heparin-induced osteoporosis is unclear. Heparin has been shown to inhibit collagen synthesis in organ culture systems, but has also been reported to stimulate collagen synthesis in osteoblast cultures. Moreover, heparin has been shown to augment PTH-stimulated bone resorption in organ cultures and to interact with unknown serum factor(s) to stimulate bone resorption by disaggregated osteoclasts. In the present study, we report that heparin, in the absence of PTH or serum, can stimulate the process of bone resorption. To our knowledge, this is the first report of heparin alone, in the absence of exogenous factors, stimulating the process of bone resorption in an organ culture system.

Sulfation was found to be a major determinant of heparin's ability to effect bone resorption. While heparin (2.5 to 3 sulfate groups per hexosamine) exerted a strong stimulatory effect on bone resorption, the ability of N-desulfated heparin to stimulate bone resorption was greatly reduced. Similarly, the less sulfated glycosaminoglycan, dermatan sulfate (1.2 sulfate groups per hexosamine) had no effect. This later observation is of particular significance because dermatan sulfate, which catalyzes thrombin inhibition by heparin cofactor II, has been used clinically with less hemorrhagic complications than unfractionated heparin. Sulfation has also been shown to be a major determinant in heparin's ability to inhibit collagen synthesis in fetal rat calvaria and to interact with unknown serum factors to stimulate bone resorption by disaggregated osteoclasts.

Size was also found to be a major determinant of the ability of heparin to effect bone resorption. Thus, the commercially available LMWHs were found to produce significantly less calcium loss from bone than unfractionated heparin. Moreover, when compared with unfractionated heparin, much higher LMWH concentrations were required before maximum isotope release was observed. In agreement with our findings, size was also suggested to be a determinant in heparin's ability to stimulate the activity of disaggregated osteoclasts. In contrast, previous studies using fetal rat calvaria have reported that the commercially available LMWHs and unfractionated heparin decrease collagen synthesis to the same extent. Thus, although our findings suggest that LMWH preparations produce much less bone resorption than unfractionated heparin, it is unknown if LMWHs can produce osteopenia as a result of decreased rates of bone formation.

Our results are at variance with other experimental studies, but are consistent with recent clinical reports. Other studies using mouse calvaria in organ culture have failed to demonstrate a direct effect of heparin on bone resorption. Moreover, the results of these other studies differ from ours, as...
they report that dermatan sulfate and other glycosaminoglycans, with few or no sulfate groups, can stimulate bone resorption.30 There is no clear explanation for the discrepancy between our findings and those of the previous reports.

Data using animal models to compare the effects of low molecular weight and unfractionated heparin on bone is severely limited. In a recent study, Monreal et al35 treated rats with unfractionated heparin and fragmin and reported that both heparins decreased bone mineral density, but that the effects of fragmin were less severe. In a separate study, however, logiparin and unfractionated heparin were reported to decrease bone density to a similar extent.35 Thus, studies with two different commercially available LMWHs have yielded conflicting results. The reason(s) for these discrepant findings are unclear. However, we have observed no significant differences between the LMWHs in terms of their ability to promote 45Ca release. Moreover, our results would indicate that a significant benefit should be derived by LMWH use.

Our findings, which suggest that the risk of heparin-induced osteoporosis may be reduced by the use of LMWHs, are consistent with limited clinical reports. It is well known that unfractionated heparin can cause spontaneous fractures of the rib or vertebrae,7,11,12 and there are anecdotal reports of the successful use of a LMWH in patients.34 The first properly designed randomized trials addressing the relative effects of heparin and a LMWH on clinical osteoporosis was reported recently by Monreal et al.35 These investigators compared fragmin (5,000 IU anti-Xa sc subcutaneously twice a day [sc bd]) with unfractionated heparin (10,000 IU sc bd) in 80 patients with deep vein thrombosis. Treatment was administered for a period of 3 to 6 months. Six of 40 patients who received unfractionated heparin developed spinal fractures compared with only one patient out of 40 receiving fragmin. Previously, Dahlman36 studied 184 women receiving long-term prophylaxis heparin therapy during pregnancy and reported a 2.2% incidence of vertebral fractures. The doses of unfractionated heparin and the duration of treatment were similar in both studies, but patients in the Monreal study were older.

Table 1. Effect of Sulfation on Heparin’s Ability to Promote 45Ca Release

<table>
<thead>
<tr>
<th>Glycosaminoglycan</th>
<th>Concentration (μg/mL)</th>
<th>45Ca Release (T/C ratio)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heparin</td>
<td>1</td>
<td>1.14 ± 0.04</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>1.53 ± 0.08*</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>1.49 ± 0.04*</td>
</tr>
<tr>
<td>N-desulfated heparin</td>
<td>1</td>
<td>1.07 ± 0.08</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>1.08 ± 0.12*</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>1.27 ± 0.12*</td>
</tr>
<tr>
<td>Dermatan sulfate</td>
<td>1</td>
<td>0.99 ± 0.04</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>0.92 ± 0.04</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>0.93 ± 0.03</td>
</tr>
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*P < .01 when compared with the release of isotope from calvaria cultured in the presence of media alone.
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In summary we used a well-described rat calvaria model to compare unfractionated heparin with four LMWHs in an attempt to determine the possible benefits of LMWH use. The LMWHs were found to produce significantly less calcium loss than unfractionated heparin. These findings are consistent with the limited clinical studies that suggest that the risk of heparin-induced osteoporosis may be reduced by the use of LMWHs.

REFERENCES

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