Repeated immobilization stress reduces rat vertebral bone growth and osteocalcin

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Received 27 December 1999; accepted in final form 7 September 2000

Patterson-Buckendahl, Patricia, Milan Rusnák, Koki Fukuhara, and Richard Kvetnánský. Repeated immobilization stress reduces rat vertebral bone growth and osteocalcin. Am J Physiol Regulatory Integrative Comp Physiol 280: R79–R86, 2001—We previously showed that psychological stressors alter plasma levels of osteocalcin (pOC), a bone-specific mineral binding protein, in ways that differ with the type of stressor. To determine effects of chronic stress, we examined vertebrae, pOC, and corticosterone levels from conscious rats subjected to foot-restraint immobilization (Immo) daily for 1–42 times. After 40–42 Immo, basal pOC was decreased by 25% compared with unstressed rats, and the subsequent rise in pOC during Immo was blunted. Corticosterone was elevated 10-fold during Immo. Immo for seven times did not change vertebral OC concentration, but caused a slight decrease in calcium and phosphorous concentrations in younger rats. Rats Immo for 42 times exhibited reduced body weight, vertebral weight, and vertebral OC concentration but no significant differences in vertebral mineral concentrations. Body fat content was visibly decreased. We do not know the source of or the stimulus for the initial rise in pOC. We conclude that both decreased growth and bone OC concentration are due to repeatedly elevated stress hormones.

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IMMOBILIZATION REDUCES BONE GROWTH AND OSTEOCALCIN

(18). These hormones normally prepare the body to react to a threat of danger by heightening arousal, blood flow, and/or muscular activity. The Immo model caused a rapid and marked increase in pOC, significant within 5 min and maximally 50–100% above basal levels after 20–30 min (33). Selective elimination of the hormonal influences by surgical removal of the adrenal medulla (elimination of adrenal catecholamines), the entire adrenal (elimination of adrenal catecholamines and corticosteroids), or by chemical blockade of release of hormones at the level of sympathetic ganglia (NE or others) showed that both corticosterone and sympathetic neural function, but not adrenomedullary hormones, were required for pOC to return to basal levels. Immobilization repeated daily for 7 days blunted but did not eliminate the pOC response. The consistency of the acute elevation of pOC during Immo suggests that it may be a very important component of an animal’s response to severe stress.

Because repeated imposition of Immo appeared to blunt the pOC response, we therefore wished to determine whether there would be a corresponding effect on bone. Previous studies had indicated a correlation between pOC and bone OC concentrations. For example, both microgravity of spaceflight and ground-based simulation studies of microgravity decreased bone growth rate as well as OC concentration in the third lumbar vertebrae (30, 31). On the other hand, supplementation with vitamin D, which stimulates OC synthesis, resulted in an increased accumulation of vertebral bone OC, but no change in bone weight (29). The current study describes the effects of 2-h Immo repeated daily for up to 42 times on pOC and on bone growth and accumulation of OC and mineral in the third lumbar vertebra.

METHODS

Immobilization stress was carried out by methods described by Kvetnansky and Mikulaj (18). The stress procedure was reviewed and approved by the Animal Care Committees of the National Institutes of Health-National Institute of Neurological Diseases and Stroke (experiments 1 and 2) and the Institute for Experimental Endocrinology, Slovak Academy of Sciences (experiment 3) before the current series of experiments. Briefly, each animal was restrained in a prone position on an immobilization board for periods not exceeding 120 min. Its head was restricted from movement by a metal loop over the nose, and its feet were taped to raised supports with bandage tape. This type of immobilization is a standardized procedure, which results in well-characterized catecholamine and corticosteroid responses. Male Sprague-Dawley-derived rats were obtained from Taconic Farms, Germantown, NY (experiments 1 and 2), or Charles River Laboratories, Wiga, Germany (experiment 3). Rats were housed four to a cage under vivarium conditions of a 6 AM to 6 PM light cycle, with food and water provided ad libitum. Experiments began at least 7 days after arrival, and animals in each experimental group were weight matched (range 280–320 g) at the beginning of the experiment.

For repeated Immo, rats were removed daily from their cages, immobilized for 2 h, and returned to their cages. Immo took place between 7 AM and 12 noon. For experiments 1 and 2, Immo was repeated 5 days a week with 2 days’ rest in between until the final week of the experiments, when Immo took place every day. Maximum number of Immo was 40 (experiment 1) or 42 times (experiment 3).

Experiment 1 provided blood samples to compare 40 times Immo rats (40 × n = 10) to 1 time Immo (1 ×, n = 12). To allow repeated blood sampling during Immo, each rat had a cannula placed into the tail artery under pentobarbital sodium anesthesia 24 h before the last Immo. Blood was collected from the cannula before Immo and after 5, 20, 60, and 120 min of Immo and replaced with equal volumes of isotonic saline containing 50 units heparin/ml. Trunk blood was obtained from all other rats after decapitation.

Experiment 2 compared absolute (never stressed) controls with one time (1 ×), six times with 24 h recovery (6 ×), and seven times (7 ×) Immo rats; all groups consisted of seven rats each. Six rats served as previously stressed, adapted controls for 7 × rats for evaluation of recovery from Immo. Absolute control and 6 × adapted control rats were killed by decapitation on removal from their cages; 1 × and 7 × Immo rats were killed immediately on removal from the Immo board.

Experiment 3 was similar to experiment 2, but also included a group of 1 × Immo allowed to recover for 24 h (1 × adapted), one group Immo for 41 times and allowed to recover for 24 h (41 × adapted), and one group Immo for 42 times (42 ×). The animals that were allowed to recover for 24 h after the last Immo served as previously stressed controls for acute 1 ×, short-term (7 ×), and long-term (42 ×) Immo killed immediately after 120 min Immo for evaluation of recovery from Immo. Animals were weighed twice a week to monitor growth; all animals were killed on the same day.

Third lumbar vertebrae were dissected from rats in experiments 2 and 3 after decapitation, cleaned of soft tissue, and freeze-dried to constant weight. Bones were then ground to a fine powder in a liquid nitrogen-cooled mill (Spen Industries, Edison, NJ). Duplicate aliquots of each bone powder were extracted for 24 h with 0.5 M ammonium EDTA, pH 8, containing protease inhibitors, in Eppendorf tubes turned end over end at 4°C to solubilize OC (12). Mineral concentrations were determined on duplicate samples (1–1.5 mg) of bone powder extracted for 24 h with 100 μl concentrated HCl. Plasma and bone OC were assayed by the RIA method of Patterson-Allen et al. (28), with guinea pig anti-rat OC antibody and purified rat OC for standard and 125I-labeled tracer. Inter- and intra-assay variations were 8.6 and 6.2%, respectively. Sensitivity for rat OC is 0.3 ng/ml of sample. The average variation of OC determination in duplicate bone samples was 3.4%. Calcium concentrations were determined by atomic absorption spectrophotometer (Perkin-Elmer) and inorganic phosphorus by the ammonium molybdate method (Sigma reagents). The average variation of duplicate bone samples was 1.9% for calcium and 2.2% for phosphorus. Plasma corticosterone was determined with commercial RIA reagents from ICN. Intra-assay variation was 3.4%. All samples from one experiment were analyzed in the same assay.

Data are presented as means ± SE. Plasma and weight gain data were analyzed for significant effects using SAS for Macintosh General Linear Models procedure for repeated-measures ANOVA. Other data were analyzed by one-way ANOVA. Duncan’s multiple-range test with least squared means comparisons was used for post hoc analysis to determine significant differences between groups. Significance level was set at P < 0.05.
RESULTS

Experiment 1. Figure 1 is a comparison of plasma OC levels in 1× Immo rats compared with those of rats that were restrained 2 h daily for a total of 40 times (40×) and killed after the last Immo. These data show that elevation of pOC during Immo is blunted, but not abolished, by long-term repetition. Analysis of between-subjects effects indicated highly significant differences in the response of the two groups (P < 0.001). Duncan’s multiple-range test showed that 1× rats were significantly different from 40× rats at each of the time points (P < 0.01), with basal pOC level in repeatedly stressed rats being ~25% below that of the 1× rats before the onset of Immo (P < 0.01). After 20 min Immo, the maximal increase in 1× rat pOC was ~50% above basal value (P < 0.001), whereas 40× rats increased by only 40% (P < 0.01). After 120 min, both groups were ~40% lower than their respective levels at the beginning of Immo (P < 0.01 for both groups).

Experiment 2. Table 1 gives data for pOC and vertebral weight and composition from animals that were subjected to Immo repeated daily for up to seven times. (6× rats that had been allowed to recover for 24 h after their last Immo served as previously stressed controls for 7× rats for evaluation of recovery from and possible adaptation to Immo.) Duncan’s multiple-range test showed that pOC levels of both 6× and 1× rats killed immediately after first Immo were not different from absolute (never stressed) controls. However, pOC in rats killed after 120 min of a seventh Immo was significantly different from all other groups (P < 0.01). They were 30% below that of absolute controls and 33% lower than 6× adapted controls (P < 0.001). There were no differences in the vertebral weights among the four groups of rats, nor did bone OC concentrations differ. Mineral analysis did show slight but significant differences between control and Immo rats. Calcium concentrations in all Immo groups were lower by ~5% in controls (P < 0.001), and phosphorous concentration was lower in the 6× adapted controls and 7× groups compared with absolute controls (P < 0.05 and 0.001, respectively) and was also different between 6× and 7× (P < 0.05). Calcium-to-phosphorous ratios in Immo groups were also significantly lower than in controls. Total bone content of calcium in 1× vertebrae was

Table 1. Plasma OC, weight, and concentration and total content of calcium, phosphorus, and OC in third lumbar vertebrae of rats subjected to 2 h foot restraint Immo daily for 1–7 times compared with unstressed (absolute control) or 6 times Immo rats allowed to recover for 24 h (6× adapted control)

<table>
<thead>
<tr>
<th></th>
<th>Absolute Control</th>
<th>1× Immo</th>
<th>6× Adapted Control</th>
<th>7× Immo</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma OC, ng/ml</td>
<td>209 ± 5</td>
<td>210 ± 23</td>
<td>216 ± 8</td>
<td>145 ± 8b,a</td>
</tr>
<tr>
<td>Vertebral wt, mg</td>
<td>135 ± 4</td>
<td>129 ± 4</td>
<td>132 ± 5</td>
<td>132 ± 2</td>
</tr>
<tr>
<td>OC, μg/mg</td>
<td>2.73 ± 0.11</td>
<td>2.84 ± 0.06</td>
<td>2.67 ± 0.04</td>
<td>2.69 ± 0.09</td>
</tr>
<tr>
<td>Ca, μg/mg</td>
<td>254 ± 2</td>
<td>243 ± 2</td>
<td>243 ± 2b,d</td>
<td>238 ± 2c,d</td>
</tr>
<tr>
<td>P, μg/mg</td>
<td>113 ± 5</td>
<td>113 ± 0.5</td>
<td>111 ± 0.6a,d</td>
<td>109 ± 0.6a,d</td>
</tr>
<tr>
<td>Ca/P, ratio</td>
<td>2.24 ± 0.1</td>
<td>2.15 ± 0.11</td>
<td>2.19 ± 0.02</td>
<td>2.18 ± 0.01a</td>
</tr>
<tr>
<td>OC, μg</td>
<td>370 ± 25</td>
<td>366 ± 11</td>
<td>352 ± 13</td>
<td>351 ± 13</td>
</tr>
<tr>
<td>Ca, mg</td>
<td>34.1 ± 1.2</td>
<td>31.3 ± 0.8a</td>
<td>32.2 ± 0.8</td>
<td>31.6 ± 0.7</td>
</tr>
<tr>
<td>P, mg</td>
<td>15.2 ± 0.5</td>
<td>14.6 ± 0.4</td>
<td>14.7 ± 0.4</td>
<td>14.3 ± 0.3</td>
</tr>
</tbody>
</table>

All values are means ± SE. Significant difference from absolute controls is indicated as follows: aP < 0.05; bP < 0.01; cP < 0.001. d and e indicate significant differences of P < 0.05 and P < 0.001 between adapted control (6×) and 7× foot restraint immobilization (Immo). OC, osteocalcin.
significantly less than in absolute controls (P < 0.05), but 6× and 7× vertebrae did not differ from absolute controls or from each other. There were no differences in total phosphorus or OC contents of these bones.

**Experiment 3.** This experiment compared effects of both short-term repetition (7×) and long-term repetition of Immo (42×) with acute (1×) and untreated (absolute) controls. As in experiment 2, rats immobilized for the same number of times but allowed to recover for 24 h before being killed served as previously stressed controls for evaluation of recovery from and possible adaptation to Immo. It should be noted that these rats were 7 wk older at the end of the experiment than those in experiment 2, when both growth rate and bone formation rate have begun to decrease. Figure 2 depicts weight gained by the respective groups over the 56-day experimental period, with black bars indicating the entire period of Immo for each repeatedly Immo group. Absolute controls and both 1× groups were identically treated until the last experimental day, and there were no statistical differences in body weights at any time; therefore, data for these three groups were combined. Duncan’s multiple-range test showed no significant differences in weight among control and 1× or 7× rat groups until the 7× group was Immo toward the end of the experimental period. By the seventh Immo of this group, the animals had actually lost weight and were 11% lighter than control and 1× Immo rats (P < 0.001). Rats in the 42× Immo group had diverged from the other animals by the 10th experimental day (P < 0.001) and weighed an average of 18% less than controls at the end of the experiment (P < 0.001). We attribute most of this difference to a visibly decreased body fat in the repeatedly Immo rats noted but not quantified during necropsy.

Figure 3 shows pOC and corticosterone levels at the end of experiment 3. Rats that were immobilized one time had pOC equal to absolute control rats immediately after 120 min Immo or after 24 h recovery. Duncan’s multiple-range test showed that rats killed immediately after 120 min of the seventh Immo had significantly lower pOC than absolute controls (P < 0.05), but did not differ from short-term adapted controls that were 6× Immo and had a 24-h recovery.
Table 2. Body weight, freeze-dried weight, OC, calcium and phosphorous composition of third lumbar vertebrae of rats subjected to 2 h Immo daily for up to 42 times, compared with previously unstressed (absolute) controls and with adapted controls allowed to recover for 24 h after 1, 6, or 41 daily Immo

<table>
<thead>
<tr>
<th></th>
<th>Absolute Control (n = 8)</th>
<th>1× Adapied Control (n = 8)</th>
<th>1× Immo (n = 8)</th>
<th>6× Adapied Control (n = 8)</th>
<th>7× Immo (n = 8)</th>
<th>41× Adapied Control (n = 9)</th>
<th>42× Immo (n = 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body wt, g</td>
<td>506 ± 15</td>
<td>509 ± 13</td>
<td>521 ± 14</td>
<td>443 ± 16†</td>
<td>473 ± 7</td>
<td>410 ± 13‡</td>
<td>424 ± 10‡</td>
</tr>
<tr>
<td>Vertebra wt, mg</td>
<td>239 ± 6</td>
<td>245 ± 6</td>
<td>245 ± 7</td>
<td>233 ± 0.010</td>
<td>259 ± 8</td>
<td>214 ± 10*</td>
<td>216 ± 6*</td>
</tr>
<tr>
<td>Vertebral wt, mg/g body wt</td>
<td>0.473 ± 0.008</td>
<td>0.483 ± 0.001</td>
<td>0.472 ± 0.013</td>
<td>0.526 ± 0.011†</td>
<td>0.547 ± 0.011‡</td>
<td>0.552 ± 0.003†</td>
<td>0.510 ± 0.004*</td>
</tr>
<tr>
<td>OC, µg/mg</td>
<td>2.43 ± 0.12</td>
<td>2.39 ± 0.06</td>
<td>2.64 ± 0.07‡</td>
<td>2.44 ± 0.07</td>
<td>2.51 ± 0.06</td>
<td>1.98 ± 0.07‡</td>
<td>2.00 ± 0.02‡</td>
</tr>
<tr>
<td>Ca, µg/mg</td>
<td>235 ± 2</td>
<td>238 ± 2</td>
<td>236 ± 2</td>
<td>232 ± 2</td>
<td>239 ± 2</td>
<td>232 ± 2</td>
<td>236 ± 4</td>
</tr>
<tr>
<td>Pi, µg/mg</td>
<td>103 ± 1</td>
<td>102 ± 2</td>
<td>104 ± 1</td>
<td>103 ± 2</td>
<td>103 ± 1</td>
<td>102 ± 1</td>
<td>99 ± 2</td>
</tr>
<tr>
<td>OC, µg/bone</td>
<td>580 ± 31</td>
<td>585 ± 20</td>
<td>648 ± 22§</td>
<td>568 ± 26</td>
<td>650 ± 25§</td>
<td>420 ± 14‡</td>
<td>430 ± 14‡</td>
</tr>
<tr>
<td>Ca, mg/bone</td>
<td>56.1 ± 1.4</td>
<td>58.3 ± 1.6</td>
<td>58.0 ± 1.6</td>
<td>54.0 ± 2.1</td>
<td>61.8 ± 1.8§</td>
<td>49.8 ± 2.6*</td>
<td>50.8 ± 1.8*</td>
</tr>
<tr>
<td>Pi, mg/bone</td>
<td>24.6 ± 0.6</td>
<td>25.0 ± 0.9</td>
<td>25.6 ± 0.8</td>
<td>23.9 ± 0.9</td>
<td>26.6 ± 0.9§</td>
<td>21.8 ± 1.1*</td>
<td>21.4 ± 0.8†</td>
</tr>
</tbody>
</table>

All values are means ± SE. Vertebral weight is also shown relative to body weight. OC and minerals are given as concentration and total bone content. Significant difference from absolute controls is indicated as follows: †P < 0.05; ‡P < 0.01; §P < 0.001. † indicates P < 0.05 between Immo and the respective adapted control allowed to recover 24 h. Period before being killed. Animals killed after 42 Immo had pOC ~16% below absolute controls (P < 0.05), whereas rats killed 24 h after the 41st Immo had pOC 25% below absolute controls (P < 0.05). This confirms observations in experiment 1 that repeated Immo impairs the ability to restore normal levels of pOC. Corticosterone levels in all rats killed immediately after Immo were elevated at least 10-fold over absolute controls (P < 0.001), but had returned to relatively normal levels in all rats killed after 24 h recovery from Immo.

Table 2 shows body and bone weights and OC, calcium, and phosphorus concentration and content in third lumbar vertebrae from rats in experiment 3. Combined data for vertebral weight from the two groups of long-term Immo rats (41× and 42×, which did not differ significantly from each other) indicated a 12% lower bone weight compared with control and 1× Immo rats (P < 0.001). When adjusted for body weight, vertebrae of both short-term and long-term repeatedly Immo rats were heavier than absolute controls, indicating a greater deficit in body than in skeletal weight gain.

Concentrations of OC but not mineral in bones of long-term Immo rats differed from controls. OC concentrations in these vertebrae were decreased by 18% compared with absolute controls. Least squares means analysis yielded significance of P < 0.001 versus all other groups. There was a significant difference in OC between 1× Immo and 1× adapted control vertebrae (P < 0.05). Total vertebral bone OC was 27% lower in 42× Immo rats than in absolute controls (P < 0.001). Significantly greater content of OC was found in both 1× and 7× Immo relative to their adapted controls killed 24 h after Immo (P < 0.05). There were no differences in mineral concentrations in these bones. Total content of calcium and phosphorus in the vertebrae was decreased in 7× and in 41× and 42× rats (P < 0.05) compared with absolute controls in proportion to their lower bone weight. There was a greater content of both minerals in 7× than in 6× adapted controls (P < 0.05).

**DISCUSSION**

Foot-restraint Immo of conscious rats causes a rapid increase in plasma levels of stress hormones and is accompanied by an equally rapid increase in pOC (33). As shown in Fig. 1, the acute increase of pOC in animals repeatedly subjected to this stressor is blunted, but not eliminated. We have now analyzed samples from more than 15 experiments comparing a variety of treatments, including surgical ablation of hormones, sympathetic blockade, cold exposure, and repeated Immo, and in all cases to date, pOC was acutely increased within the first 20 min of Immo (Ref. 33 and unpublished observations). Furthermore, we have detected significantly increased pOC within the first 3 min of Immo (Patterson-Buckendahl et al. unpublished observations). Repeated sampling over a 120-min period of rats similarly cannulated but not subjected to Immo showed no change from baseline levels (Ref. 33 and unpublished observations). pOC is clearly affected by the physiological response to stress and may be an integral part of that response.

We do not as yet know whether Immo-elevated pOC results from increased osteoblastic secretion or whether it might be coming from the bone surface by a noncellular mechanism. Hypocalcemia induced by citrate or EDTA infusion was accompanied acutely by both increased parathyroid hormone and pOC in a time course similar to what we observed with Immo (10, 11, 37). A similar response was seen in monkeys anesthetized with isoflurane, but not with ketamine or ketamine plus atropine (13). It has been suggested that the acute rise in OC in these induced hypocalcemias...
originates from the bone surface rather than from new synthesis or secretion (11). Induction of de novo synthesis is much less likely, due to the extreme rapidity of the response. It has also been suggested that the elevated OC may somehow be mediating the hypocalcemia that results from these treatments (2).

Repetitive foot-restraint Immo markedly altered the rate of increase in body weight. Animals that were Immo toward the end of experiment 3, when growth rate in controls had actually slowed, exhibited an 11% decrease in body weight over the 7-day period, but no change in vertebral weight. The two groups whose Immo began 8 wk earlier gained weight, but at a decreased rate, and by the end of the experiment had gained 18% less than 1× and control rats. A major factor in these differences in body weight is the decreased amount of body fat in the repeatedly Immo animals (personal observations). It has been long established that elevated glucocorticoids as well as Epi mobilize energy stores to enable animals to respond to acute severe stressors. Furthermore, Marti et al. (20) reported decreased food intake in repeatedly Immo animals. Michajlovskij et al. (21) reported reduced food and water intake by rats Immo for 150 min daily for 7 days. The greatest change in consumption followed the first Immo, and there was a gradual increase over the next 6 days. When normalized to body weight, consumption of food and water after a sixth Immo did not differ from the control pre-Immo period, suggesting adaptation of subsequent food consumption to the Immo stress procedure (21). The combined effects of decreased food intake and increased fat mobilization could explain the decreased weight gain of Immo rats. Our animals were housed in group cages to avoid the added stressful influence of isolation, thus we could not directly evaluate individual food intake.

A further effect of repeated Immo was seen in the lower weight of the third lumbar vertebrae. Some of this decrease may be attributed to decreased body weight, which is well known to affect ultimate bone weight. The physiological responses to decreased food intake, resulting in decreased body weight, are complex and include changes in growth hormone, insulin, thyroid hormone, leptin, and many other factors. Many of these factors also directly influence bone metabolism. Recently, leptin (a hormone produced by adipose tissue) was shown to affect bone mass via a hypothalamic pathway (7). We have not measured leptin in Immo rats, but would expect it to be altered if food intake was decreased.

It is also likely that repeated high corticosterone levels were a major factor in the decreased bone growth as well as in decreased weight gain. The myriad direct and indirect effects of glucocorticoids on bone were well summarized by Lukert and Kream (19). Among the specific bone parameters affected are collagen and OC synthesis, as well as differentiation of bone-forming osteoblastic cells. Furthermore, intestinal uptake of calcium is diminished, thus reducing the supply of mineral for deposition into bone in growing animals. Chronic exogenous glucocorticoids are often associated with severe osteoporosis in humans. Although most studies of osteoporotic effects involve pharmacological doses of steroids far higher than those reached even under severely stressful conditions, chronic low doses administered by inhalation have also been shown to decrease bone density (14). Moderate to high endogenous cortisol levels in humans (within the range of corticosterone in our rats) with Cushing’s disease or with major depression also result in osteoporosis (22).

Our data are the first we are aware of to document the effects of chronic immobilization stress and the associated endogenous elevations of stress hormones on skeletal growth.

The biochemical makeup of the third lumbar vertebrae was also altered. In younger rats (weight range 280–300 g) subjected to Immo for seven times (experiment 2, Fig. 2), the mineral makeup of bone was altered, but not the OC concentration. At this age, OC synthesis rate and osteoblastic activity are high and decrease markedly during the next 2 mo (12) and may override the effects of Immo. The magnitude of the mineral decrease is consistent with the percentage of bone mineral believed to be readily exchangeable. This could result from decreased dietary intake (20) as well as impairment of intestinal calcium absorption. Other investigators have reported decreased plasma calcium in response to foot-restraint Immo (23). It seems probable that this mineral, which is crucial to muscle and nerve function, would be replaced from a ready supply in bone that is not dependent on cellular activation.

We cannot explain the ~10% greater concentration of OC in 1× Immo rats killed immediately compared with those killed after 24 h of recovery (adapted controls). Neither group was significantly different from the absolute controls that had never been subjected to Immo. Both sets of animals were housed and handled identically until 1× rats were subjected to Immo at the end of the experimental period. Other investigators have estimated a 5-min half-life for pOC clearance (35). It seems unlikely that all of the acutely elevated pOC could be scavenged at that rate by the one vertebra analyzed. Even if it were, it would still not account for the differences between rats killed immediately after 120 min Immo and their adapted control Immo rats on the previous day. On the assumption of an elevation of pOC from the control level of ~70 ng/ml and a plasma volume of 6% of body weight, there should have been ~2 μg pOC in circulation. Given an acute twofold elevation such as we have seen in previous experiments (33) and a 5-min half-life, ~30 μg would be cleared during 120 min Immo. This is still less than the 63 μg difference between 1× Immo rats and their adapted or absolute controls. It is also unlikely that synthesis was increased during Immo, given the high corticosterone levels of the rats. The nonsignificant increase in vertebral weight of 7× Immo compared with 6× adapted or absolute control rats could account for the differences in total bone content of OC, calcium, and phosphorous in those animals.

In experiment 3, elevated pOC levels in 1× and 7× Immo rats were followed by recovery to control levels.
after 24 h. It should be noted that there is a delay in the effects of elevated corticosterone on pOC of 1.5–4 h (26, 32). This would explain the observation that pOC during or at the end of Immo may be increased in the presence of very high corticosterone levels, yet markedly decreased after 2 h. However, both basal plasma levels and bone OC were significantly decreased in older, chronically Immorats. The combined effects of the stress hormones are important in restoring pOC levels to baseline values, because in the absence of corticosterone or sympathetic neural input (NE), pOC levels remain highly elevated (33). The effect of repeated Immo on tyrosine hydroxylase, the rate-limiting enzyme in the biosynthetic pathway leading to NE, is to decrease mRNA expression and enzyme activity (27, 36). Because decreased NE allows an increase in OC (33), this adaptive response would tend to blunt the post-Immo decrease in pOC, even though elevated corticosterone acted to decrease OC synthesis. Any short-term effect on the OC in bone of 1× and 7× rats would be small and masked by the high concentration already incorporated. After 8 wk of repeated Immo, bone OC concentration was decreased by 20% in vertebrae that were also lighter by 12% than those of control or 1× rats. It is likely that the repeatedly elevated corticosterone in these animals impaired osteoblast function, synthesis of OC, and the corresponding accumulation of OC in bone over the 8-wk duration of the experiment. If, as others have suggested, the pOC originates on the bone surface, then the pool of bone OC available to respond to subsequent Immo would be diminished. Our data support this possibility.

The role of OC in bone has not been definitively elucidated. It has been implicated in control of both resorption and formation. Experiments of Glowacki et al. (9) showed decreased resorption of OC depleted bone particle implants. Ducy et al. (8) showed that bone in mice whose OC genes were deleted and that contained no detectable OC grew normally, was stronger and more dense, and had smaller mineral crystals than bones of wild-type mice (8). These data suggested possible autocrine regulation of osteoblast function by OC. This possibility was recently confirmed by identification of putative receptors for OC on osteoblasts (3). Thus decreased bone OC content of chronically Immorats might be accompanied by a change in mineral quality rather than concentration, due to a combination of decreased resorption and decreased formation. This could provide a protective mechanism for their decreased quantity of bone. It will be important in future work to determine whether decreased OC in response to repeated Immo leads to changes in mineral density distribution.

The authors thank Slavka Gorcikova for assistance with immobilization and Dale Buckendahl for assistance with bone preparation and editing of the manuscript. We thank Larissa Pohorecky, Arthur Tomie, and Greg Blakley for helpful discussion of the manuscript. Work was supported in part by National Institute on Alcohol Abuse and Alcoholism R21-AA-12705–01 (to P. Patterson-Buckendahl) and Slovak Grant Agency for Science 2–610999 (to R. Kvetnansky).

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