Rest-inserted loading rapidly amplifies the response of bone to small increases in strain and load cycles

Sundar Srinivasan,1 Brandon J. Ausk,1 Sandra L. Poliachik,1 Sarah E. Warner,1 Thomas S. Richardson,2 and Ted S. Gross1

Departments of 1Orthopaedics and Sports Medicine and 2Statistics, University of Washington, Seattle, Washington

Submitted 2 May 2006; accepted in final form 25 January 2007

The attempt to use physical activity as a means of augmenting bone mass in humans has been principally governed by the concept that more loading (i.e., higher strains, higher strain rates) induces a greater response (21, 24). This thesis is supported by numerous in vivo studies that have examined a wide range of independent and dependent mechanical parameters (e.g., strain magnitude and rate, frequency, cycle number, strain gradients). These studies, without exception, have demonstrated that an increased stimulus begets increased bone formation, bone mass, and bone strength (7, 14, 18, 22, 25, 29, 32).

However, this relation is not linear, as a threshold level of stimulus must be reached for mechanical loading to induce an adaptive response by the tissue (13). As evidenced in other fields (9, 11, 26), examining tissue responses to loading over a narrow range at/or near the threshold (i.e., peri-threshold) could prove extremely valuable in identifying cellular mechanisms underlying how small perturbations in stimuli cause abrupt changes in adaptive responses. While peri-threshold stimuli have the additional advantage of being physiologically relevant, the induced adaptive responses tend to be both nonlinear and noisy. Adding complexity, the threshold behavior to mechanical loading in bone is dependent on a number of components of the induced mechanical environment. For example, the threshold of strain magnitude required to initiate bone formation is dependent on the frequency of the loading waveform as well as the number of loading cycles (7, 25, 30, 36). An additional nonlinearity arises as the response of bone to mechanical loading rapidly saturates. At the tissue level, this saturation of bone’s response is clearly demonstrated by the lack of additional response induced when cycle numbers are increased while strain magnitude is held constant (30, 37).

We recently demonstrated that removing 10 of every 11 load cycles of a 1-Hz repetitive loading waveform (i.e., allowing the tissue to “rest” at zero load for 10 s between each load cycle) transformed a low-magnitude regimen that was ignored by the tissue into one that dramatically enhanced bone formation (34). In this context, insertion of a rest interval appeared to lower the “threshold” magnitude at which bone formation could be initiated. Subsequently, a number of groups have observed that rest-inserted loading is highly stimulatory in a variety of models and experimental conditions (2, 19, 27, 33, 38).

The counterintuitive efficacy of rest-inserted loading suggests that brief rest intervals may fundamentally alter how bone cells perceive and respond to mechanical loading. We therefore hypothesized that a 10-s rest interval (at zero load) inserted between each load cycle would increase the osteogenic effects of mechanical loading near previously identified thresholds for strain magnitude and cycle numbers. We tested our hypothesis by subjecting the right tibiae of female C57BL/6J mice (16 wk, n = 70) to exogenous mechanical loading within a peri-threshold physiological range of strain magnitudes and load cycle numbers using a noninvasive murine tibia loading device. Bone responses to mechanical loading were determined via dynamic histomorphometry. More specifically, we contrasted bone formation induced by cyclical vs. rest-inserted loading (10-s rest at zero load inserted between each load cycle) by first varying peak strains (1,000, 1,250, or 1,600 με) at fixed cycle numbers (50 cycles/day, 3 days/wk for 3 wk) and then varying cycle numbers (10, 50, or 250 cycles/day) at a fixed strain magnitude (1,250 με). Within the range of strain magnitudes tested, the slope of periosteal bone formation rate (p.BFR/BS) with increasing strain magnitudes was significantly increased by rest-inserted compared with cyclical loading. Within the range of load cycles tested, the slope of p.BFR/BS with increasing load cycles of rest-inserted loading was also significantly increased by rest-inserted compared with cyclical loading. In sum, the data of this study indicate that inserting a 10-s rest interval between each load cycle amplifies bone’s response to mechanical loading, even within a peri-threshold range of strain magnitudes and cycle numbers.
not receive exogenous loading, served as the contralateral control. Normal cage activity was allowed between loading sessions, and food and water were provided ad libitum. Animals received calcine labels (15 mg/kg ip) administered on days 10 and 19 and were killed on day 22 via CO2 inhalation. The University of Washington Animal Care and Use Committee approved all experiments.

**Strain magnitude study.** Young adult female C57BL/6J mice (16 wk) were randomly assigned to one of six groups (n = 7 per group). Mice received either 50 cycles/day of cyclic loading (1-Hz trapezoidal waveform, 0.01 s⁻¹ strain rate) or 50 cycles of loading (same trapezoidal waveform and strain rate) with a 10-s rest interval (at zero load) inserted between each load cycle at three different load magnitudes. On the basis of calibration studies (described below), the mice received external loading sufficient to induce 1,000-, 1,250-, or 1,600-με peak longitudinal normal strains at the periosteal surface of the tibia midshaft. Strain rates were held constant across groups, with dwell time periods at peak strain adjusted to achieve the three peak-induced strain magnitudes (0.8, 0.75, 0.68 s, respectively).

**Load cycle number study.** Female C57BL/6J mice (16 wk) were randomly assigned to one of four groups (n = 7 per group) and received cyclic loading or rest-inserted loading (as described above) sufficient to induce 1,250-με peak periosteal longitudinal normal strains at the tibia midshaft. Mice received either 10 or 250 load cycles per day. Mice receiving 50 cycles of cyclic and rest-inserted loading at 1,250 με from the strain magnitude threshold study were also included in this analysis.

**Calibration of induced strain environment.** As previously described, a combined strain-gauging and finite element (FE) modeling approach was used to calibrate the normal strains induced in the tibia diaphysis by the loading device (15, 33). Immediately post death, two uniaxial strain gauges (Texas Measurements, College Station, TX) were attached via cyanoacrylate to the lateral and medial surfaces of the right tibia midshaft of adult female C57BL/6J mice (16 wk, n = 2). The calibration mice were positioned within the loading device, and right tibia was loaded for five sequential load cycles over a range of loading magnitudes (0.1–0.5 N, random order). Five trials were performed at each load magnitude (with mice removed from the loading device between each trial). Strain-gauge signals were amplified (2120A amplifier, Vishay Measurement Groups, Raleigh, NC), and induced strains were recorded using a DAPView program (Microstar Laboratories, Bellevue, WA). High-resolution μCT scans (μCT20, Scanco Medical, Bassersdorf, Switzerland) of the tibia and fibula were then obtained (18 μm per voxel resolution). FE models of the tibia and fibula structure were generated from the serial scans using custom multimodule automated software (developed within PV-Wave). With the use of previously published bone material properties (4), the boundary conditions produced in the noninvasive mouse tibia loader were simulated in the FE model, solved using Patran (MSC, Los Angeles, CA) and strain distributions in the tibia diaphysis of each of the calibration mice were determined. Over the range of loads considered, the induced strains exhibited a linear relation to applied loads. The FE predictions were within 4% (range 1–9%) of strain-gauge data at the strain-gauge sites. From these data, a peak induced strain vs. load calibration curve was established. On the basis of this curve, external loads in the range of 0.225 to 0.375 N were sufficient to yield peak periosteal longitudinal normal strains in the range of 1,000–1,600 με.

Postdeath, animals-specific peak longitudinal normal strains were determined for all loaded mice in the strain magnitude study using beam theory (33, 34). Briefly, given the strain distributions determined at the tibia midshaft from the validated FE model of our calibration experiments, we used beam theory to calculate the force and moment boundary conditions at the tibia midshaft. The animal-specific strain distributions induced at the midshaft were then determined by applying the force/moment boundary conditions to the mirrored contralateral tibia midshaft section of each mouse enrolled in the strain magnitude study.

**Histomorphometry.** At death, the right experimentally loaded (termed “loaded”) and the contralateral left (termed “control”) tibiae were dissected of soft tissue, fresh sectioned and a cross section (300 μm) spanning the tibia midshaft was obtained using a minitome (Stuers, Copenhagen, Denmark). Sections were hand ground to 90 μm, placed under a coverslip, and imaged using a Nikon Epifluorescent microscope. Digital images were obtained, analyzed blinded, and dynamic and static histomorphometry measures were determined at the endocortical and periosteal surfaces using custom-written software within PV-Wave (VNI, Boulder, CO). Briefly, single-labeled (sLS), double-labeled surfaces (dLS), and interlabel thickness (Ir.L.Th) were measured, and surface referent mineralizing surface [MS/BS = (0.5 • s.LS + d.LS)/BS], mineral apposition rate (MAR = Ir.L.Th/Ir.L.t, where Ir.L.t was the time in days between labels), and bone formation rate (BFR/BS = MS/BS • MAR) were determined per standard histomorphometry (12, 23). Please note that prefixes “e” and “p” represent abbreviations for measures at the endocortical and periosteal surfaces, respectively. Static histomorphometry measures, including cortical area (Ct.Ar), periosteal envelope (Ps.Ar), endocortical envelope (Ec.Ar), and cortical thickness (Ct.Th) were also determined (34).

**Statistical analysis.** The primary anticipated outcome measure of the experimental design was p.BFR/BS. Linear regression analysis indicated that the relation between p.BFR/BS in experimental vs. contralateral control was not significant in either the strain magnitude or load cycle study (maximum r² = 0.08). As a result, analysis of response measures in experimentally loaded and contralateral controls was performed on absolute data (rather than relative measures of bone formation).

Primary statistical analyses were performed via factorial ANOVAs. To compare peak strain magnitudes in the strain magnitude study, a 3 × 2 factorial ANOVA was performed (i.e., contrasting animal specific strains in the 1,000-, 1,250-, and 1,600-με groups for cyclic vs. rest-inserted loading). For analysis of dynamic histomorphometry measures at the endocortical (and periosteal) surface, separate 3 × 2 ANOVAs were performed for MS/BS, MAR, and BFR/BS in experimentally loaded and in contralateral control bones to detect if the main effects of the three strain magnitudes (or 3-load cycle numbers) and the two protocol types (cyclic vs. rest inserted) significantly influenced response measures.

If main effects for a particular response measure (e.g., p.BFR/BS) in experimentally loaded bones were found to be significant, then post hoc analysis for the response measure was performed when the following two criteria were satisfied: 1) the main effects were not statistically significant in contralateral controls and 2) the linear regression between the residuals of the response measure in loaded bones vs. the residuals of response measure in control bones did not attain statistical significance. The latter criterion identified response measures where basal activity in experimentally loaded bones was not correlated with basal activity in contralateral tibiae and permitted additional analysis of that subset of absolute data. In both the strain magnitude and load cycle study, the first criterion was satisfied for all measures at both bone surfaces. The second (residual) criterion was violated for e.MS/BS and e.BFR/BS in both strain magnitude and load cycle studies and for p.MS/BS in the load cycle study. This subset of data was therefore not further analyzed.

For the remaining response measures, the following three secondary post hoc analyses were performed: 1) a paired t-test was performed to determine if loading induced responses significantly different from contralateral controls; 2) a Tukey honestly significant difference test was performed to examine if response measures were significantly different across strain magnitudes (or load cycle numbers); and 3) an independent t-test was performed to examine if response measures were significantly different between rest-inserted vs. cyclic loading. Lastly, as surface BFR is the ultimate arbiter of whether a loading regimen was osteogenic, we performed the following regressions. First, we performed a linear regression to examine if the slope of p.BFR/BS as a function of increasing...
strain magnitudes (or load cycle numbers) was different from zero. Regressions of the form: \( \text{p.BFR/BS} = \alpha + \lambda \times y \) were used, where \( y \) was the induced strain (or load cycle number). If the parameter \( \lambda \) attained significance, then the slope of BFR was considered significantly different from zero. Next, to compare \( \text{p.BFR/BS} \) across loading waveforms, we used a regression of the form: \( \text{p.BFR/BS} = \alpha + \beta \times R + \lambda \times y + \delta \times y \times R \), where \( R \) takes on values 0 or 10-s rest. If the parameter \( \delta \) attained significance, we concluded that the slope of \( \text{p.BFR/BS} \) as a function of increasing strain magnitudes (or load cycle numbers) of rest-inserted loading was significantly different from that for the cyclic loading protocol. A similar statistical analysis was performed for the static histomorphometry measures. For all comparisons, \( P \leq 0.05 \) was considered to be statistically significant. All results are reported as means ± SD.

RESULTS

In both the strain magnitude and load cycle number studies, there were no main effects on the static histomorphometry measures. Specifically, the main effects did not attain significance for Ct.Ar, Ec.Ar, Ps.Ar, or Ct.Th in experimentally loaded bones (data not shown). Of particular interest as a surrogate measure for osteoclastic activity, there was also no significant increase in Ec.Ar between experimentally loaded and control bones across the different groups.

Strain magnitude study: At both the endocortical and periosteal surfaces, induced peak strain magnitudes were significantly elevated as load magnitudes were increased (Table 1; \( P < 0.001 \) for all measures attaining significance). At the same applied load, no statistical differences were observed in peak strain magnitudes induced in the cyclic loading compared with rest-inserted loading groups (range of differences: 3.0–8.6%).

The influence of loading regimens on endocortical osteoclastic activity was subtle (Table 2). No main effects were observed for any response measure in control bones. In experimentally loaded bones, main effects were significant for both e.MS/BS (\( P = 0.01 \)) and e.BFR/BS (\( P = 0.05 \)). However, the residual response criterion was violated in both cases (\( P < 0.01, 0.03 \), respectively), and post hoc analyses were therefore not performed.

At the periosteal surface, the influence of loading regimens on bone response measures was more pronounced (Fig. 1). Main effects for all dynamic response measures were not significant in control bones. In contrast, main effects were all significant in experimentally loaded bones, with only one exception (the main effect of protocol type on p.MS/BS, \( P = 0.12 \)). Residual criteria were not violated for any measure. As a result, all post hoc analyses were possible with the exception of protocol type (i.e., cyclic vs. rest) on p.MS/BS.

Compared with contralateral control bones, both cyclic and rest-inserted loading elevated periosteal osteoblast activity. The observed response was dependent on both the magnitude of induced strain and the type of loading (Fig. 1). At 1,000 \( \mu \text{e} \) peak strain, while cyclic loading significantly increased only p.MS/BS (\( P = 0.01 \)), rest-inserted loading significantly increased both p.MS/BS (\( P = 0.03 \)) and p.BFR/BS (\( P = 0.05 \)). At 1,250–\( \mu \text{e} \) peak strain, while cyclic loading significantly increased p.MS/BS (\( P = 0.02 \)) and p.BFR/BS (\( P = 0.03 \)), rest-inserted loading elevated all response measures (\( P < 0.01, 0.04, 0.02 \) for p.MS/BS, p.MAR, and p.BFR/BS, respectively). At 1,600-\( \mu \text{e} \) peak normal strain, both cyclic and rest-inserted loading significantly elevated all dynamic response measures compared with contralateral controls (\( P \leq 0.01 \)).

For the cyclic loading group, increased strain magnitude minimally influenced periosteal osteoblast activity. Only p.MS/BS induced at 1,600-\( \mu \text{e} \) peak strain was significantly increased compared with that induced by the 1,000-\( \mu \text{e} \) cyclic loading protocol (Fig. 1). In contrast, rest-inserted loading at 1,600 \( \mu \text{e} \) significantly elevated p.MS/BS, p.MAR, and p.BFR/BS compared with that observed in the 1,250-\( \mu \text{e} \) rest-
At 1,250- 0.001 for all measures; Fig. 1). Differential effects were also noted (vs. contralateral bones (*), vs. cyclic loading (†), and vs. 1,000 ‡) or 1,250 increased p.MAR (‡). The observed in the 1,000- rest-inserted loading group demonstrated significantly increased p.MAR (P = 0.001) and p.BFR/BS (P = 0.02) compared with cyclic loading.

Within the range of strains studied, the slope of p.BFR/BS for cyclic loading was not significantly different than zero (r² = 0.17, P = 0.06). In contrast, the slope of p.BFR/BS induced by rest-inserted loading was significantly different from zero (r² = 0.62, P < 0.001). Finally, the slope of p.BFR/BS for rest-inserted loading was significantly elevated compared with cyclic loading (P = 0.05; Fig. 2).

Load cycle number study. When strain magnitude was held constant, the response at the endocortical surface varied with increasing cycle numbers but was confounded by variability in baseline activity (Table 3). While main effects for each dynamic response measure (i.e., e.MS/BS, e.MAR, e.BFR/BS) were not significant in control bones, they were significant for all measures in experimentally loaded bones (P < 0.001 for all measures). However, the residual criterion was violated for e.MS/BS (P < 0.01) and e.BFR/BS (P = 0.04). As a result, post hoc analyses were only possible for e.MAR.

Compared with contralateral bones, both cyclic (P = 0.03) and rest-inserted loading elevated e.MAR at 250 cycles/day (P = 0.001, Table 3). While e.MAR in experimental bones subject to cyclic loading was not increased as a function of load cycles, e.MAR induced by 250 cycles/day of rest-inserted loading was significantly elevated compared with that induced by 10 cycles/day of rest-inserted loading. Additionally, e.MAR induced by 250 cycles/day of rest-inserted loading was significantly increased compared with cyclic loading (P < 0.001).

At the periosteal surface, bone’s response to increasing numbers of load cycles was robust. The main effects for each dynamic response measure (i.e., p.MS/BS, p.MAR, p.BFR/BS) were not significant in control bones. The main effects were significant for all measures in experimentally loaded bones (P < 0.01 for all measures). However, the residual criterion was violated for p.MS/BS (P = 0.03). As a result, post hoc analysis of p.MS/BS was not performed.

Both cyclic and rest-inserted loading elevated periosteal osteoblastic activity compared with contralateral controls (Fig. 3). Specifically, cyclic loading at both 50 (P = 0.03) and 250 cycles/day (P = 0.04) significantly increased p.BFR/BS vs. controls (p.MAR was not elevated by any of the cyclic loading protocols; Fig. 3). In contrast, rest-inserted loading significantly increased both p.MAR and p.BFR/BS at 10, 50,
and 250 cycles/day compared with controls ($P < 0.01$ for all measures).

Across the range of load cycles studied, an increased number of cyclic loading cycles minimally influenced osteoblastic activity (Fig. 3). Specifically, when animals were subject to cyclic loading, neither p.MAR nor p.BFR/BS was significantly different across groups when animals were subject to 10, 50, or 250 cycles/day. In contrast, p.MAR induced by 250 cycles/day of rest-inserted loading was significantly increased compared with that induced by 10 cycles/day ($P = 0.01$). Furthermore, p.BFR/BS induced by 250 cycles/day of rest-inserted loading was significantly increased compared with both 10 ($P = 0.001$) and 50 cycles/day ($P = 0.03$). Rest-inserted loading distinctly elevated periosteal osteoblastic activity compared with cyclic loading. At 50 cycles/day, rest-inserted loading significantly increased p.MAR compared with cyclic loading ($P < 0.01$, Fig. 3). At 250 cycles/day, rest-inserted loading significantly increased both p.MAR ($P < 0.01$) and p.BFR/BS ($P < 0.01$) compared with 250 cycles/day of cyclic loading.

Within the range of load cycles studied, the slope of p.BFR/BS induced by cyclic loading was not significantly different from zero ($r^2 = 0.06, P = 0.27$). In contrast, the slope of p.BFR/BS induced by rest-inserted loading was significantly different from zero ($r^2 = 0.5, P < 0.001$). Finally, the slope of p.BFR/BS for rest-inserted loading was significantly increased compared with that induced by cyclic loading ($P = 0.01$; Fig. 4).

DISCUSSION

In this study, the right tibiae of young adult female C57BL/6J mice were subjected to either cyclic or rest-inserted mechanical loading. Osteoblastic responses on the endocortical and periosteal surfaces were examined in response to increases in peak strain magnitude (with cycle number held constant at 50 cycles/day) or cycle numbers (with peak strain held constant at 1,250 $\mu$e). The range of both variables were designed to span the peri-threshold region required for cyclic loading to induce elevated periosteal bone formation on the basis of previous reports in the literature (7, 13). We found that rest-inserted loading was osteogenic (i.e., significantly increased p.BFR/MS) at the lowest peak strain magnitudes and load cycles considered in this study, while cyclic loading was not. Compared with cyclic loading, rest-inserted loading significantly enhanced the slope of p.BFR/BS with increasing strain magnitudes and loading cycle numbers.

The experimental design implemented in this study purposefully explored the behavior of bone in response to perithreshold levels of mechanical loading. As in other biological and nonbiological systems (9, 11, 26), stimulus thresholds must be surpassed for mechanical stimuli to induce an osteogenic response in bone (13). Given the similarity of peak strain magnitudes and frequency spectra induced by functional activity in a variety of species (1, 5, 20), the achievement of bone homeostasis may be based, in part, on cellular recognition of basal levels of mechanical stimulus that do not provoke an adaptive response, as well as recognition of stimuli that require adaptation (3, 28). Investigating whether rest intervals at zero load serve to heighten bone’s sensitivity to mechanical stimuli with the peri-threshold homeostatic range, in our view, may provide a unique opportunity to identify the mechanisms underlying the effectiveness of rest-inserted loading. For example, at this “level” of mechanical stimulus in our experimental model (15), only portions of the cortex experience induced strains within the peri-threshold range. As such, the possibility that cortical osteocytes/lining cells/osteoblasts signaling pathways will be activated in some portions of the cortex, but not in others within the same cross section could provide a powerful means to screen for pathways activated by rest-inserted loading.

While this long-term goal guided our study, our experimental design does incorporate several limitations. Retrospectively, group sizes in our study ($n = 7$/group) were somewhat small for the implemented statistical approaches. However, post hoc data analysis indicated that increasing group size to $n = 9$/group would not alter our primary conclusions. Second, randomization of animals into experimental groups resulted in assignment of few mice with comparatively higher endocortical basal activity in some but not all of the groups. This variability resulted in violation of the residual criterion for the majority of response measures at the endocortical surface. Regardless, this study was designed to induce strains near the peri-threshold level at the periosteal surface and anticipated activation of bone formation at that surface. Given the mode of applied loads, induced maximal endocortical strains were well below this level ($\sim$30% lower strain magnitudes) and were not anticipated to be sufficient to induce a pronounced response (7, 13). A final limitation lies with the potential influence that longer anesthesia durations had on bone responses in the rest-inserted loading groups particularly in the cycle number

### Table 3. Endocortical MS/BS, MAR and BFR/BS as a function of increasing load cycle numbers

<table>
<thead>
<tr>
<th></th>
<th>Cyclic</th>
<th>Rest Inserted</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10 cycles/day</td>
<td>50 cycles/day</td>
</tr>
<tr>
<td></td>
<td>10 cycles/day</td>
<td>50 cycles/day</td>
</tr>
<tr>
<td>c.MS/BS, %</td>
<td>5.8±7.2</td>
<td>18.9±10.0</td>
</tr>
<tr>
<td></td>
<td>4.2±3.6</td>
<td>26.8±6.8</td>
</tr>
<tr>
<td>c.MAR, μm/day</td>
<td>0.27±0.12</td>
<td>0.38±0.08</td>
</tr>
<tr>
<td></td>
<td>0.34±0.07</td>
<td>0.43±0.10</td>
</tr>
<tr>
<td>e.BFR/BS, μm²/μm²·day⁻¹</td>
<td>0.02±0.03</td>
<td>0.08±0.05</td>
</tr>
<tr>
<td></td>
<td>0.02±0.02</td>
<td>0.12±0.05</td>
</tr>
<tr>
<td></td>
<td>5.7±6.4</td>
<td>12.9±14.6</td>
</tr>
<tr>
<td></td>
<td>16.8±12.2</td>
<td>18.6±12.9</td>
</tr>
</tbody>
</table>

Endocortical MS/BS, MAR, and BFR/BS as a function of increasing load cycle numbers of cyclic or rest-inserted loading at magnitude sufficient to induce 1,250 $\mu$e peak longitudinal normal strains at the periosteal surface (mean ± SD). Significant differences in response measures from contralateral control bones (*), from cyclic loading (†), and from 10 cycles/d (‡) are noted.

[53x256]and 50 cycles/day (P with that induced by 10 cycles/day (P of rest-inserted loading was significantly increased compared with controls (P < 0.01 for all measures). Across the range of load cycles studied, an increased number of cyclic loading cycles minimally influenced osteoblastic activity (Fig. 3). Specifically, when animals were subject to cyclic loading, neither p.MAR nor p.BFR/BS was significantly different across groups when animals were subject to 10, 50, or 250 cycles/day. In contrast, p.MAR induced by 250 cycles/day of rest-inserted loading was significantly increased compared with that induced by 10 cycles/day (P = 0.01). Furthermore, p.BFR/BS induced by 250 cycles/day of rest-inserted loading was significantly increased compared with both 10 (P = 0.001) and 50 cycles/day (P = 0.03). Rest-inserted loading distinctly elevated periosteal osteoblastic activity compared with cyclic loading. At 50 cycles/day, rest-inserted loading significantly increased p.MAR compared with cyclic loading (P < 0.01, Fig. 3). At 250 cycles/day, rest-inserted loading significantly increased both p.MAR (P < 0.01) and p.BFR/BS (P < 0.01) compared with 250 cycles/day of cyclic loading.

Within the range of load cycles studied, the slope of p.BFR/BS induced by cyclic loading was not significantly different from zero (r² = 0.06, P = 0.27). In contrast, the slope of p.BFR/BS induced by rest-inserted loading was significantly different from zero (r² = 0.5, P < 0.001). Finally, the slope of p.BFR/BS for rest-inserted loading was significantly increased compared with that induced by cyclic loading (P = 0.01; Fig. 4).

The experimental design implemented in this study purposefully explored the behavior of bone in response to perithreshold levels of mechanical loading. As in other biological and nonbiological systems (9, 11, 26), stimulus thresholds must be surpassed for mechanical stimuli to induce an osteogenic response in bone (13). Given the similarity of peak strain magnitudes and frequency spectra induced by functional activity in a variety of species (1, 5, 20), the achievement of bone homeostasis may be based, in part, on cellular recognition of basal levels of mechanical stimulus that do not provoke an adaptive response, as well as recognition of stimuli that require adaptation (3, 28). Investigating whether rest intervals at zero load serve to heighten bone’s sensitivity to mechanical stimuli with the peri-threshold homeostatic range, in our view, may provide a unique opportunity to identify the mechanisms underlying the effectiveness of rest-inserted loading. For example, at this “level” of mechanical stimulus in our experimental model (15), only portions of the cortex experience induced strains within the peri-threshold range. As such, the possibility that cortical osteocytes/lining cells/osteoblasts signaling pathways will be activated in some portions of the cortex, but not in others within the same cross section could provide a powerful means to screen for pathways activated by rest-inserted loading.

While this long-term goal guided our study, our experimental design does incorporate several limitations. Retrospectively, group sizes in our study (n = 7/group) were somewhat small for the implemented statistical approaches. However, post hoc data analysis indicated that increasing group size to n = 9/group would not alter our primary conclusions. Second, randomization of animals into experimental groups resulted in assignment of few mice with comparatively higher endocortical basal activity in some but not all of the groups. This variability resulted in violation of the residual criterion for the majority of response measures at the endocortical surface. Regardless, this study was designed to induce strains near the peri-threshold level at the periosteal surface and anticipated activation of bone formation at that surface. Given the mode of applied loads, induced maximal endocortical strains were well below this level (~30% lower strain magnitudes) and were not anticipated to be sufficient to induce a pronounced response (7, 13). A final limitation lies with the potential influence that longer anesthesia durations had on bone responses in the rest-inserted loading groups particularly in the cycle number.
cycles focused on using the strategy to transform low-magni-
sity cyclic loading regimens otherwise ignored by bone into osteogenic stimuli (33, 34). Subsequent studies have suggested
that rest intervals may add further benefit to cyclic loading regimens that are already stimulatory for bone formation (19,
27, 38). The data presented here further support these obser-
vations. In groups in which cyclic loading did not significantly
alter periosteal bone formation (e.g., 50 cycles at 1,000 με and
10 cycles at 1,250 με), insertion of 10-s rest intervals caused
significantly enhanced p.BFR/BS. Additionally, rest intervals
served to additionally enhance the potency of already osteo-
genic cyclic loading protocols (e.g., 50 cycles/day at 1,600 με
and 250 cycles/day at 1,250 με).

In a broader context, our data suggest that bone’s response to
increasing cyclic and rest-inserted strain magnitudes were
clearly differentiated. In previous studies examining a wide
range of strain magnitudes (e.g., 1,000–4,000 με), the peri-
osteal response to increased magnitude stimuli manifests as an
enhanced adaptive response (16, 31). However, in our study,
the slope of periosteal bone formation with increasing strain
magnitudes of cyclical loading was not significant from zero and
was poorly correlated with strain magnitude (there was a
trend for the slope being non-zero). While this result appears
contradictory given previous studies, this result most likely
arose as our study examined bone formation within a relatively
narrow range of strain magnitudes. In clear contrast, the re-
sponse of bone to rest-inserted loading was highly correlated
with the magnitude of applied strains. Additionally, the slope
of periosteal bone formation with increasing strain magnitudes
of rest-inserted loading was significantly elevated compared
with cyclic stimuli. Taken together, these data indicate that
cyclic stimuli within this narrow range provides a minimal
adaptive stimulus, consistent with loading within a peri-thresh-
old range and of magnitudes similar to that encountered during
normal functional activity (20).

Our data also provide a context to examine saturation of
adaptive response to a given stimulus, a phenomenon observed
in a variety of cell and tissue types (6, 8). A hallmark of
saturation is the lack of additional or increased response to a
repeated stimulus that was initially stimulatory—a pheno-
menon well recognized in bone (30, 37). It has been hypothesized
study. Compared with the cyclic loading groups (maximum of
6 min), two rest-inserted loading groups were exposed to
anesthesia for longer durations (50 cycle: 9.1 min; 250 cycle:
46 min). Two factors limit the impact of this limitation. First,
the mice were exposed to anesthesia three times per week.
Second, if the length of anesthesia were to confound our
results, it would be expected to reduce osteoblastic activity.
Yet, the longest duration anesthesia group (250 cycle/day
rest-inserted loading) still demonstrated significant elevations
in bone formation compared with cyclic loading and vs. groups
subject to fewer cycle numbers of rest-inserted loading.

Our initial studies with rest intervals inserted between load
cycles focused on using the strategy to transform low-magni-
that the desensitization or accommodation of bone cells to mechanical stimuli underlies the saturation of adaptive responses at the scale of bone tissues (30, 35). Consistent with this fundamental characteristic of bone adaptation to increasing loading cycles (i.e., response saturation), periosteal bone formation rates induced by cyclic loading were not altered by increases in number of load cycles. In clear contrast, p.BFR/BS induced by rest-inserted loading was significantly related to increasing load cycle numbers within the range considered (i.e., 10–250 cycles/day) and with a slope substantially different from the response elicited by cyclical mechanical stimuli. However, it is currently unclear whether bone’s response to rest-inserted loading has also saturated within this range of load cycles. This issue will prove challenging to resolve as 250 cycles of rest-inserted loading already requires a substantial anesthesia period. Nevertheless, our data suggest that inserting rest between load cycles delays saturation of bone formation response and broadens the range of load cycle numbers over which enhanced adaptation might be achieved.

These data do provide some clues regarding the mechanisms underlying the efficacy of rest-inserted loading. Given that strains (and load cycles) were similar across loading type (i.e., rest inserted vs. cyclic), the data also suggest that the differential adaptation induced by rest-inserted stimuli are more likely dependent on biophysical factors secondary to tissue deformation rather than tissue deformation directly. This study examined osteoblast function-dependent outcomes measured at the tissue level over the course of 3 wk. Acute in vitro experiments suggest that osteoblast Ca2+ signaling is responsive to alterations in stimuli magnitudes and also displays a desensitized phenotype when subject to continual physical stimuli over the course of a few minutes (10, 17). Interestingly, recent in vitro data suggest that rest-inserted fluid flow induces sustained and repeated Ca2+ signaling by individual cells compared with cyclic fluid flow (2). Rest intervals therefore appear to have profound influence at early stages of mechanotransduction by serving to “delay” cell desensitization to stimuli over the course of seconds and minutes and, given our data, may potentially alter cell sensitivity to stimuli over the course of days and weeks.

In summary, we explored the effect of inserting 10-s zero-load rest intervals between load cycles on two fundamental characteristics of bone’s response to loading within a peri-threshold range of strain magnitudes and cycle numbers. We observed that rest-inserted loading elicited a significant periosteal osteoblast response to small increases in applied strain—interventions that were otherwise barely stimulatory when applied without rest intervals. As such, in addition to the known influence of increases in strain magnitude, strain rates, frequency and load cycle number, inserting rest intervals (at zero load) between load cycles directly influence the osteogenic potential of mechanical loading regimens. Interestingly, the phenomenon of response saturation that is observed in bone across a variety of response measures appears to be delayed by rest insertion between load cycles. These data, taken together, suggest that rest intervals may fundamentally alter the signaling pathways by which bone’s cellular network perceives and responds to mechanical loading.

---

**REFERENCES**


