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Recovery periods restore mechanosensitivity to dynamically loaded bone

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Summary

Bone cells are capable of sensing and responding to mechanical forces, but mechanosensitivity begins to decline soon after the stimulus is initiated. Under continued stimulation, bone is desensitized to mechanical stimuli. We sought to determine the amount of time required to restore mechanosensitivity to desensitized bone cells *in vivo* by manipulating the recovery time (0, 0.5, 1, 2, 4 or 8 h) allowed between four identical daily loading bouts. We also investigated the osteogenic effectiveness of shorter-term recovery periods, lasting several seconds (0.5, 3.5, 7 or 14 s), introduced between each of 36 identical daily loading cycles. Using the rat tibia four-point bending model, the right tibia of 144 adult female Sprague-Dawley rats was subjected to bending, sham bending or no loading. In the rats receiving recovery periods between loading bouts, histomorphometric measurements from the endocortical surface of the loaded and nonloaded control (left) tibiae revealed more than 100 % higher relative bone formation rates in the 8 h recovery group than in the 0 and 0.5 h recovery groups. Approximately 8 h of recovery was sufficient to restore full mechanosensitivity to the cells. In the rats allowed time to recover between load cycles, 14 s of recovery resulted in significantly higher (66–190 %) relative bone formation rates compared to any of the three shorter recovery periods. In both experiments, bone formation in the sham-bending animals was similar to that in the nonloaded control group. The results demonstrate the importance of recovery periods for (i) restoring mechanosensitivity to bone cells and (ii) maximizing the osteogenic effects of mechanical loading (exercise) regimens.

Key words: resensitization, recovery, mechanical loading, histomorphometry, bone adaptation.

Introduction

Cells *in vivo* are routinely exposed to a wide variety of stimuli, originating from chemical, mechanical and electromagnetic sources. When challenged with a relevant stimulus for an extended period, cells undergo a process of desensitization, or adaptation, that enables them to reduce the stimulatory effects and, consequently, to maintain environmental homeostasis. Much of what is known about cellular desensitization is based on experiments addressing the biochemical stimulation of membrane receptors, in particular G-protein-coupled receptors (GPCRs). In response to prolonged agonist exposure, GPCRs can undergo (i) phosphorylation and ensuing uncoupling of the G-protein from the receptor, (ii) sequestration and internalization of the receptor from the surface and (iii) downregulation of the receptor, which requires new protein synthesis (Bünemann et al., 1999; Freedman and Lefkowitz, 1996). These mechanisms are associated with different resensitization time scales, ranging from seconds or minutes (phosphorylation) to hours or days (receptor downregulation) (Lohse, 1993).

Much less is known about cellular desensitization and resensitization in response to mechanical stimuli, which in part reflects our lack of understanding of mechanotransduction mechanisms (Duncan and Turner, 1995). In light of the known anabolic effects of mechanical loading (e.g. physical activity) on bone mass, a greater understanding of bone cell mechanodesensitization and resensitization can be used to optimize loading/exercise protocols aimed at maintaining or improving bone mass. It is reasonable to expect that, like GPCR desensitization, mechanodesensitization might occur on several different time scales and involve several different molecular mechanisms.

In vivo, bone cells respond robustly to dynamic mechanical stimuli (Robling et al., 2001; Rubin and Lanyon, 1984; Churches and Howlett, 1982; Lišková and Heřt, 1971), but their sensitivity to the stimulus wanes quickly after its initiation (Turner, 1998). Consequently, loading cycles that occur towards the end of a loading bout are not as osteogenic as those that occur towards the beginning of the bout. This phenomenon has been described as ‘diminishing returns’ (Turner, 1998), i.e. as the duration of the loading bout increases without interruption, the osteogenic response tends to saturate. The principle of mechanosensory saturation in bone cells has been

demonstrated *in vivo* in several different animal models, including the transcortically pinned turkey ulna (Rubin and Lanyon, 1984), the jumping rat model (Umemura et al., 1997) and the rat tibia four-point bending model (Turner et al., 1994a; Forwood et al., 1996). Those experiments highlight two key points about bone mechanosensitivity: (i) that mechanical loading sessions need not be long to maximize bone formation, and (ii) that extending the loading session beyond a few minutes does not contribute any additional osteogenic effect.

Implicit in the mechanosensory saturation phenomenon is the existence of a recovery period. It is clear that saturated cells do become responsive again from the observation that rat tibiae loaded once per day are capable of mounting as large a response on loading day 2 as they did on loading day 1 (Forwood and Turner, 1994; Chow et al., 1993). In a previous communication, we showed that recovery periods lasting several hours improved mechanosensitivity *in vivo* (Robling et al., 2000). Rat tibiae exposed to 360 load cycles per day exhibited greater bone formation rates if the load cycles were administered in several smaller bouts, with rest periods between bouts, than did tibiae in which all 360 cycles were given in a single, longer (uninterrupted) bout. Shorter-time-scale recovery periods also appear to be important in maximizing the osteogenic response to loading. Srinivasan and Gross (Srinivasan and Gross, 2000a) showed that rest periods lasting 10 s, introduced between individual loading cycles, enhanced the amount of surface actively forming new bone compared to bones that had been loaded for the same number of cycles but lacked a recovery period between cycles (back-to-back cycles).

Recovery periods are clearly important for restoring mechanosensitivity to desensitized bone cells, but how much time do cells require *in vivo* to become fully resensitized after a bout of loading? Using the rat tibia four-point bending model, we sought to determine the minimum amount of time required to return desensitized bone cells to their fully mechanosensitive state by manipulating the length of the recovery period between identical (90 cycles of bending, four times per day) loading bouts. We tested the hypothesis that longer interbout recovery periods result in a greater osteogenic response to loading. In a second experiment, we investigated the osteogenic effects of recovery periods on a shorter time scale, of the order of seconds, by manipulating the length of the recovery period between identical mechanical loading cycles. We hypothesized that longer intercycle recovery periods would result in a greater osteogenic response to loading.

Materials and methods

Adult female Sprague-Dawley rats (144 rats) were purchased for the experiments from Harlan, Inc. (Indianapolis, IN, USA). The rats arrived at the animal facility at Indiana University 2 weeks before the experiment began to acclimate them to their new environment. Standard rat chow and water were provided *ad libitum* during the acclimation and experimental periods. All procedures performed in the experiments were in accordance with the Indiana University Animal Care and Use Committee guidelines.

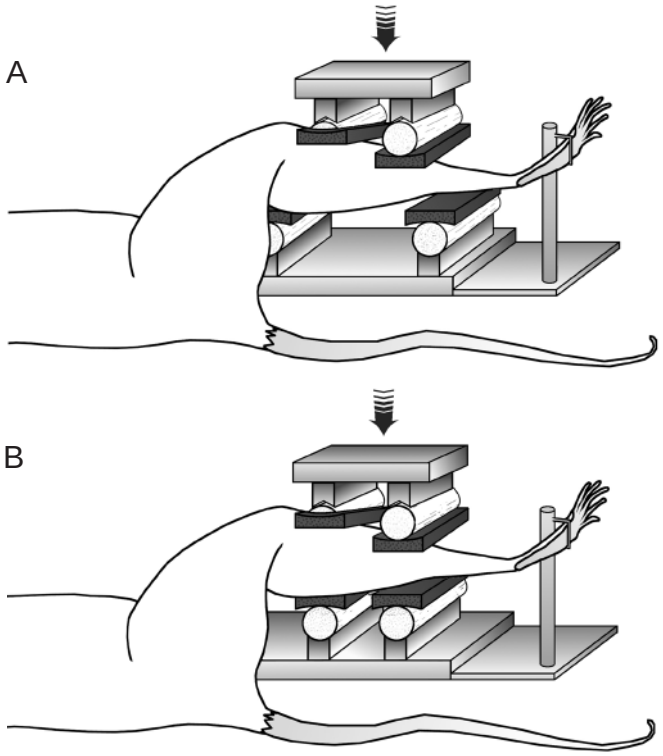

Fig. 1. Diagram of the rat tibia four-point bending apparatus with the rat *in situ*. (A) A mediolateral bending moment is produced in the portion of the tibial shaft between the two upper (padded) load points when a force is applied to the upper platen of the device. (B) By moving the lower load points inwards, so that they directly oppose the upper points, a force applied to the upper platen will squeeze the soft tissues intervening between the bone and the load points, as does the configuration depicted in A, but negligible bending of the shaft occurs. This sham configuration allows assessment of the effect of soft tissue irritation (and consequent inflammatory response) on bone formation, as distinct from a coordinated mechanically adaptive response (adapted from Robling et al., 2000).

Under ether-induced anesthesia, mechanical force was applied to the right tibia using a four-point bending apparatus (Turner et al., 1991). This system is capable of applying mediolateral bending or sham bending (periosteal pressure without bending), depending on the configuration of the load points (Fig. 1). For the rats subjected to bending, the upper load points were spaced 11 mm apart and were centered between the lower load points, which were spaced 23 mm apart (Fig. 1A). For the rats subjected to sham bending, the upper and lower sets of load points were spaced 11 mm apart and were in direct opposition to one another (Fig. 1B). In all loading groups (bending and sham bending), a peak dynamic force of 54 N was applied to the upper platen of the loading device using an open-loop, stepper-motor-driven spring linkage. When the load points are positioned for bending, 54 N elicits peak compressive strains of approximately 2400 με on the lateral periosteal surface and approximately 1300 με on the

lateral endocortical surface (Akhter et al., 1992; Turner et al., 1994b). When the load points are positioned for sham bending, negligible strains occur on the tibial surfaces (Raab-Cullen et al., 1994). The left tibia was not loaded in any of the animals and served as an internal control. All rats were allowed normal cage activity between loading bouts.

Experimental design

Long-term recovery experiment

Eighty-one rats were allocated randomly into six bending groups, two sham-bending groups and one nonloaded control group (group N=9). On each of the three loading days (days 1, 3 and 5; see Fig. 2A, top panel), the bending and sham-bending groups were administered four discrete loading bouts per day. Each bout consisted of 90 load cycles, applied as a haversine waveform at a frequency of 2 Hz. Among the bending groups, the four daily loading bouts were separated by 0, 0.5, 1, 2, 4 or 8 h of recovery time (Fig. 2A, bottom panel), during which the animals were returned to their cage. The two sham-bending groups were loaded according to the 0 and 8 h recovery schedules. The nonloaded control group received neither ether nor loading. All rats were injected intraperiotoneally with calcein (7 mg kg^{-1} body mass; Sigma Chemical Co., St Louis, MO, USA) on days 5 and 12 and were killed on day 16.

Short-term recovery experiment

Sixty-three rats were allocated randomly into four bending groups, two sham-bending groups and one nonloaded control

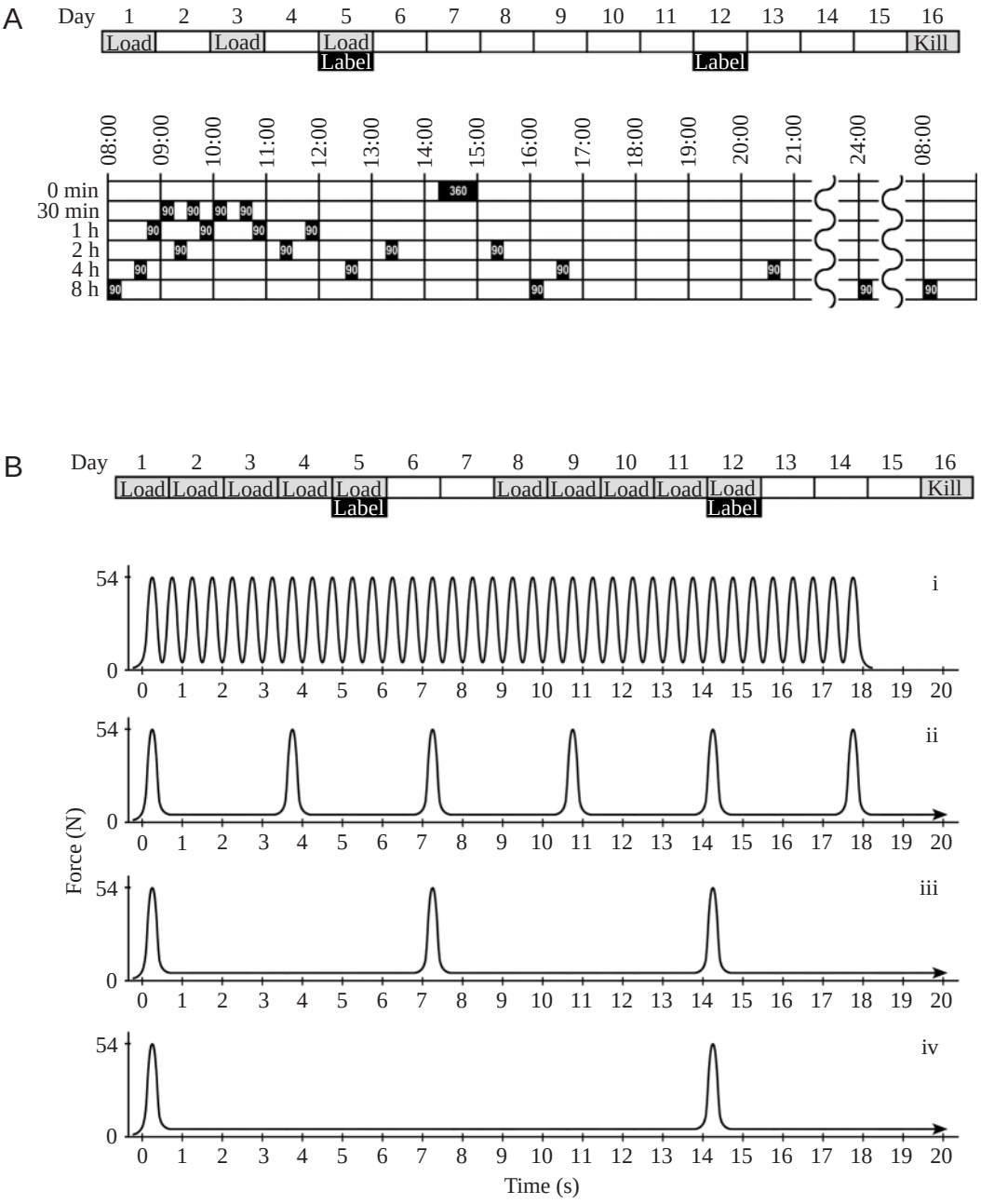

Fig. 2. Overview of the experimental design for the long-term (A) and short-term (B) recovery experiments. (A) The upper part outlines the experimental period; rats were loaded on days 1, 3 and 5 and then killed on day 16. The lower panel details a single loading day. Time of day appears across the top of the diagram. Each filled box within the diagram indicates a loading bout; the number inside the box indicates the number of cycles applied during that bout. The 0 h recovery group received a single bout of 360 cycles, which is equivalent to administering four bouts of 90 cycles with no time between each of the four bouts. Note that, at the end of each loading day, all rats (excluding those in the control group) had been given 360 load cycles. (B) The top panel outlines the experimental period; rats were loaded on days 1–5 and 8–12 and then killed on day 16. Rats were administered 36 load cycles per day with one of the following recovery periods introduced between cycles: 0.5 s (back-to-back cycles, i), 3.5 s (ii), 7 s (iii) or 14 s (iv). The 0.5 s group was loaded for 18 s (36 cycles at 2 Hz), the 3.5 s group was loaded for 2 min, the 7 s group was loaded for 4 min and 14 s group was loaded for 8 min (the total duration of the loading session is not shown on the figure for the 3.5, 7 and 14 s groups).

group (group N=9). To each of the bending and sham-bending groups, 36 load cycles were applied in a single bout on each of days 1–5 and 8–12 of the experimental period (Fig. 2B, top). The load was applied as a 2 Hz haversine waveform, pulsed every 0.5 (back-to-back cycles), 3.5, 7 or 14 s (Fig. 2Bi–iv). During the 0.5 to 14 s recovery period between pulses, the limb was retained in the loading apparatus under a 2 N static load. The two sham-bending groups were loaded according to the 0.5 and 14 s recovery schedules. The nonloaded control group received neither ether nor loading. All rats were injected intraperiotoneally with calcein (7 mg kg^{-1} body mass; Sigma Chemical Co., St Louis, MO, USA) on days 5 and 12 and were killed on day 16.

Tissue preparation and histomorphometry

After the animals had been killed, the right and left tibiae were removed immediately, cleaned of soft tissue, cleaved at the proximal and distal ends to allow proper infiltration of the marrow cavity and immersed in 10 % neutral buffered formalin for 48 h. The diaphyses were dehydrated in graded alcohols, cleared in xylene and embedded in methyl methacrylate. Using a diamond-embedded wire saw (Histo-saw; Delaware Diamond Knives, Wilmington, DE, USA), transverse thick sections (approximately 70 μm) were removed from the tibial diaphysis at a point 6 mm proximal to the tibia–fibula junction and were mounted unstained on standard microscope slides.

One section per limb was examined on a Nikon Optiphot fluorescence microscope using the Bioquant digitizing system (R&M Biometrics, Nashville, TN, USA). The following primary data were collected from the endocortical surface at 125× magnification: total perimeter (B.Pm), single-label perimeter (sL.Pm), double-label perimeter, measured along the first label (dL.Pm) and double-label area (dL.Ar). From these primary data, the following derived quantities were calculated: mineralizing surface, [MS/BS=(0.5sL.Pm+ dL.Pm)/B.Pm; %], mineral apposition rate (MAR=dL.Ar/ dL.Pm×7; μm day^{-1}) and bone formation rate [BFR/BS= MAR×(MS/BS)×3.65; $\mu m^3\,\mu m^{-2}\,year^{-1}$] (Parfitt et al., 1987). The mineralizing surface (MS) reflects the percentage of the bone surface (BS) that was actively incorporating mineral into the matrix during the labeling period. Because mineralization normally occurs in the wake of new bone formation, MS/BS reveals the fraction of pre-existing bone surface engaged in new bone formation. The mineral apposition rate (MAR) reflects the rate at which new bone was deposited in the radial direction. The bone formation rate is an overall measure of new bone formation, combining the percentage of surface actively forming new bone (MS/BS) with the radial rate of bone formation (MAR). All the derived quantities (measured from two-dimensional tissue sections) were converted into three-dimensional units using standard stereological techniques (Parfitt, 1983). The same sections used for dynamic histomorphometry were evaluated under polarized white light to determine the microstructural organization of the newly formed bone tissue.

To control for individual differences in systemic factors, left tibia (nonloaded control) values were subtracted from right tibia values; this procedure results in a new set of relative (r) values for each variable (e.g. rBFR/BS). Differences between the loaded (right) and nonloaded (left) tibiae were tested using Student's t-tests for paired variates. Differences among group means were tested for significance by analysis of variance (ANOVA), followed by Fisher's protected least significant difference (LSD) tests for all pairwise comparisons. Dunnett's method (two-tailed) was used to test for differences between the loaded groups and the nonloaded control group. A time constant describing the return of mechanosensitivity in the long-term recovery experiment was calculated using least-squares regression of $\log_2$-transformed data.

Results

Of the 144 rats used in the experiments, 12 were excluded from histomorphometric analysis because of missing labels (N=10) or anesthesia-related deaths during loading (N=2). Body mass declined slightly but non-significantly in all loaded groups ($0.25<P<0.55$) during the loading period. In both experiments, however, the length of the recovery period had no detectable effect on final body mass (P=0.58 for the long-term recovery experiment and P=0.78 for the short-term recovery experiment). Left tibia bone formation rates in each of the loaded groups were not significantly different from one another (ANOVA, $P>0.05$), which suggests that the length of the recovery period had no detectable effect on bone formation in the nonloaded skeleton, i.e. there was no systemic effect on bone formation resulting from the different recovery periods. The endocortical bone formed during the labeling period consisted exclusively of lamellar bone in both loaded and control tibiae (Fig. 3).

Long-term recovery experiment

All the bending groups exhibited a significantly greater mineralizing surface, mineral apposition rate and bone formation rate in the loaded (right) limb than in the control (left) limb, with the exception of mineral apposition rate in the 0 h group (Table 1). Sham bending resulted in non-significant right *versus* left differences for the 0 h recovery group, but the 8 h recovery sham group did exhibit a significantly greater mineralizing surface and, consequently, significantly greater bone formation rate in the loaded limb (Table 1).

Relative (right minus left) endocortical values (relative mineralizing surface, relative mineral apposition rate, relative bone formation rate) were significantly greater in each of the bending groups than in the no-load control group (Dunnett's *post-hoc*, $P<0.05$; Table 1). Neither of the sham-bending groups was significantly different from the no-load control group for any of the relative values (Dunnett's *post-hoc*, $P>0.05$; Table 1). Among the bending groups, relative mineral apposition rate showed a bending effect, but no association with recovery period duration was found (Fig. 4B). Conversely, a positive, significant trend ($P<0.001$)

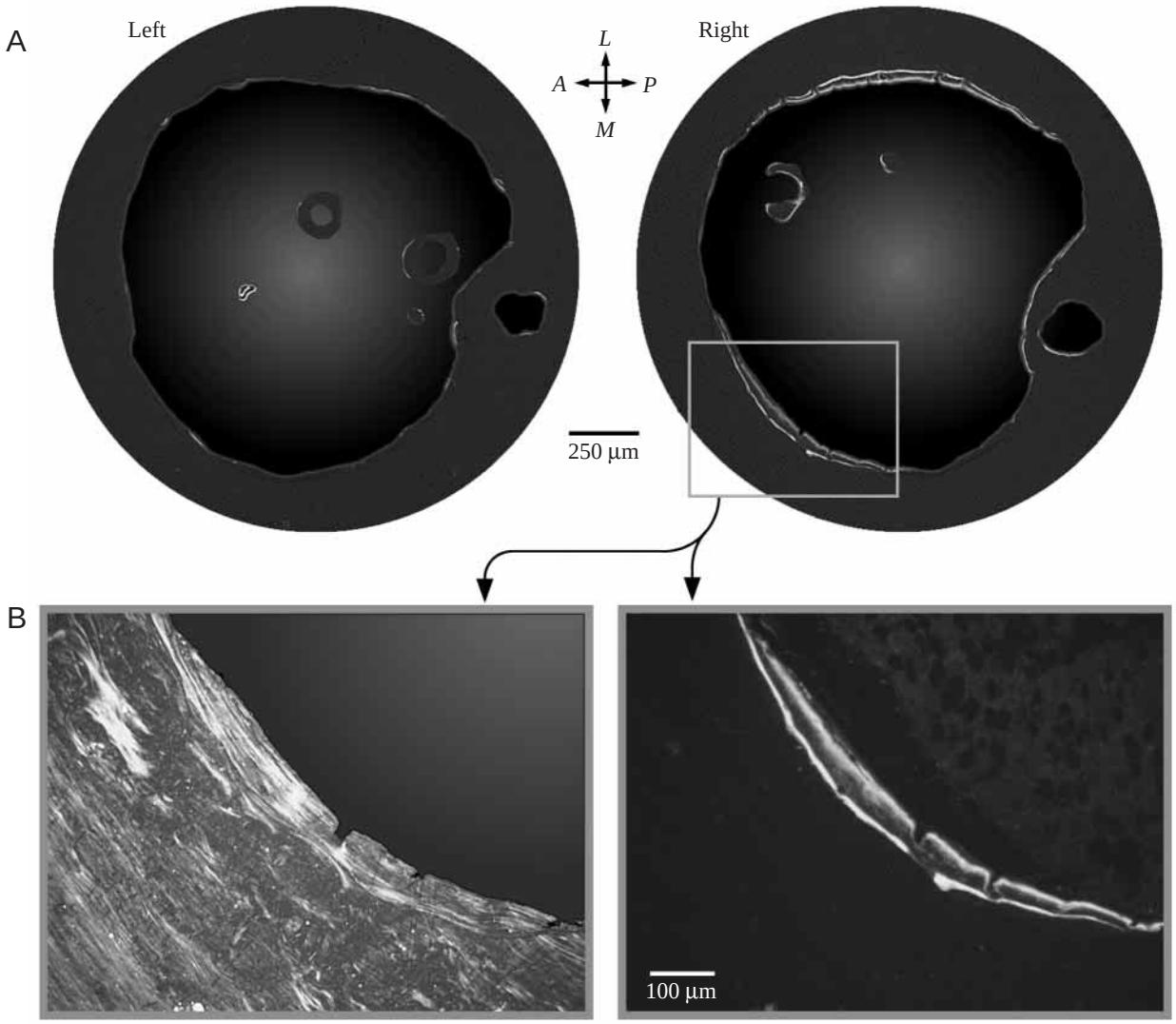

Fig. 3. (A) Fluorescence photomicrographs of the endocortical surface of a right (loaded in bending) and left (nonloaded control) tibia from an animal in the 14 s recovery group. Sparse single and double labeling is present in the control limb, but the loaded limb shows long stretches of well-separated double labeling, particularly on the medial and lateral surfaces where strains were greatest. *A*, anterior; *L*, lateral; *M*, medial; *P*, posterior. (B) Higher-power photomicrographs of the boxed area from the loaded limb in A. The left and right panels illustrate the same field under polarized white light (left panel) and fluorescence (right panel). Taken together, the photomicrographs show that the bone formed during the experiment, i.e. the bone between labels, exhibits lamellar organization.

was found between the time between bouts and each of the variables rMS/BS and rBFR/BS (Fig. 4A,C). Longer recovery periods resulted in higher relative bone formation rates. In tibias allowed 8 h of recovery between each of the four daily loading bouts, relative bone formation rates were 125 % greater than the rates found in animals that had received the four bouts with no recovery time between bouts (0 h group) and 102 % greater than the rates found in animals that had received the four bouts with a 0.5 h recovery time between bouts. Extrapolation of the recovery curve beyond 8 h revealed almost no further increase in sensitivity with increasing recovery duration (Fig. 5). Thus, a recovery period of approximately 8 h between bouts is sufficient to restore full mechanosensitivity to the cells.

Short-term recovery experiment

All the bending groups exhibited significantly greater mineral apposition rate, mineralizing surface and bone formation rate in the loaded (right) limb than in the control (left) limb (Table 2). Sham bending resulted in non-significant right *versus* left differences for the 14 s recovery group, but the 0.5 s recovery group did exhibit a significantly greater bone formation rate in the loaded limb (Table 2).

Relative (right minus left) endocortical values in the short-term recovery group were significantly greater in each of the bending groups than in the no-load control group, with the exception of relative mineral apposition rate in the 3.5 s group (Dunnett's *post-hoc*, $P<0.05$; Table 2). Neither of the sham-bending groups was significantly different from the

Table 1. *Effects of mechanical loading bout spacing (recovery period duration) on endocortical bone formation*

Time between bouts (h)	*N*	MAR (μm day^{-1}) Mean	MAR *P*-value[a]	MS/BS (%) Mean	MS/BS *P*-value	BFR/BS (μm^3 μm^{-2} year^{-1}) Mean	BFR/BS *P*-value
Bending							
0	8						
Right		2.00±0.57[b]	NS	32.96±4.18[b]	<0.01	252.16±73.45[b]	<0.05
Left		1.05±0.17		16.33±2.93		68.72±18.45	
0.5	7						
Right		2.17±0.20[b]	<0.001	31.92±3.69[b]	<0.001	250.74±33.84[b]	<0.001
Left		0.60±0.18		11.94±1.73		25.19±8.09	
1	7						
Right		1.88±0.21[b]	<0.01	49.82±5.67[b]	<0.01	357.10±72.66[b]	<0.01
Left		0.89±0.21		19.68±2.78		70.74±17.33	
2	9						
Right		2.17±0.15[b]	<0.001	46.72±4.07[b]	<0.001	357.05±26.26[b]	<0.001
Left		0.82±0.17		16.36±4.16		52.86±16.54	
4	7						
Right		2.11±0.25[b]	<0.05	59.31±3.91[b]	<0.001	442.91±38.46[b]	<0.001
Left		1.02±0.14		23.96±3.26		90.64±16.15	
8	9						
Right		2.47±0.21[b]	<0.001	49.49±5.18[b]	<0.001	466.09±72.40[b]	<0.001
Left		0.91±0.09		15.24±2.00		53.17±10.65	
Controls							
No loading	9						
Right		0.90±0.23	NS	23.63±5.43	NS	89.22±23.17	NS
Left		1.20±0.24		18.48±4.73		93.22±31.78	
0 sham	8						
Right		0.69±0.17	NS	20.33±3.96	NS	57.67±16.80	NS
Left		0.78±0.19		17.10±3.69		57.68±15.92	
8 sham	8						
Right		0.63±0.12	NS	25.29±3.84	<0.001	67.49±18.19	<0.01
Left		0.42±0.19		10.51±2.98		24.14±13.50	

Values are means ± S.E.M.

All experimental animals received mechanical loading in four bouts of 90 cycles per bout. Force was applied in a haversine waveform at 2 Hz; peak force magnitude was 54 N.

[a]Probability associated with paired *t*-test between right and left values for each group. NS (not significant) indicates that the probability exceeds 0.05.

[b]Relative mean (calculated as right minus left) is significantly different from control group relative mean at $P<0.05$ (Dunnett's *post-hoc* test).

MAR, mineral apposition rate; MS, mineralizing surface; BS, bone surface; BFR, bone formation rate.

no-load control group for any of the relative values (Dunnett's *post-hoc*, $P>0.05$; Table 2). Among the bending groups, no differences in relative mineral apposition rate were found (Fig. 6B). However, relative mineralizing surface and relative bone formation rate were significantly (66–190 %) higher in the 14 s group than in any of the three remaining bending groups (Fig. 6A,C). No significant differences were found among the 0.5, 3.5 and 7 s groups. Thus, the results suggest the existence of a short-term recovery threshold somewhere between 7 and 14 s, beyond which bone formation is enhanced over more closely spaced cycles.

Discussion

Bone cells become increasingly desensitized to load cycles applied without interruption (Rubin and Lanyon, 1984; Umemura et al., 1997). Recovery periods, during which the mechanical stimulus is withheld or drastically reduced (e.g. normal cage activity), are required to restore mechanosensitivity. Our main objective in the present study was to determine the length of the recovery period required for desensitized bone cells to regain full mechanosensitivity after a bout of loading. The data suggest that, by increasing the duration of the recovery period between loading bouts, a greater degree of sensitivity is restored to the cells.

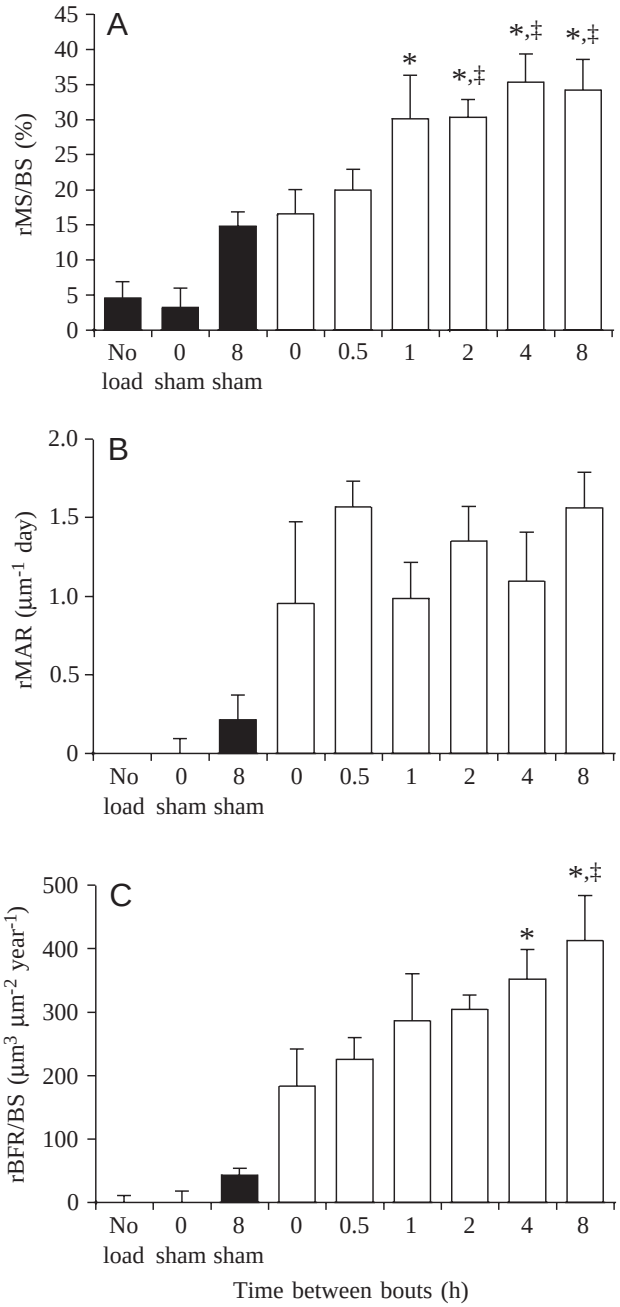

Fig. 4. (A) Relative mineralizing surface, (B) relative mineral apposition rate and (C) relative bone formation rate in the long-term recovery experiment. The relative (right minus left) bone formation rate (rBFR/BS; C) and relative mineralizing surface (rMS/BS; A) were positively associated with the recovery period duration among the bending groups (open columns). Relative mineral apposition rate (rMAR; B) exhibited a bending effect, but no trends were apparent among the bending groups. With the exception of the 8 h sham group for relative mineralizing surface, sham bending (filled columns) elicited a response similar to that observed in the nonloaded group (filled column). For comparison among bending groups, an asterisk indicates a significant difference from the 0 h bending group and a double dagger indicates a significant difference from the 0.5 h group, based on Fisher's protected LSD at α=0.05. The significance of right (loaded) *versus* left (nonloaded) comparisons is indicated in Table 1. Values are means + S.E.M. For values of *N*, see Table 1.

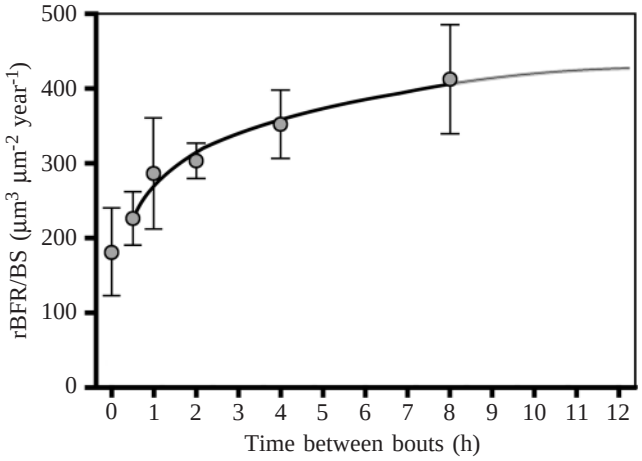

Fig. 5. Relative bone formation rate (rBFR/BS) shows essentially the same response to 8 h of recovery as would be expected from extrapolation (grey line) to 12 h of recovery, which is the maximum recovery period possible for this experimental design (spacing the bouts more than 12 h apart would have extended the first group of four bouts (load 'day' 1) into the second group of four bouts (load 'day' 2), which began 48 h after the first group of bouts began; see Fig. 2). The curve is described by the equation: rBFR/BS= 272+44.5$\log_2$(recovery time). Values are means ± S.E.M., *N*=7–9 per data point.

Approximately 8 h of recovery is sufficient to restore full mechanosensitivity, as suggested by two lines of evidence: (i) extrapolation of the recovery curve (Fig. 5) to the maximum possible recovery time possible for this experimental design (12 h) predicts no significant difference in bone formation rate from that achieved at the 8 h recovery time point and (ii) the bone formation response measured in the 8 h recovery group is of approximately the same magnitude as that observed in our laboratory when rats are loaded once every 24 h (Forwood and Turner; 1994; Turner et al., 1994b).

Among the bending groups, mechanosensitivity diminished as a result of the first loading bout of each day (Fig. 7A). In the shorter recovery period groups (e.g. 0.5 h), the second and subsequent bouts were applied before substantial resensitization had been established; thus, subsequent cycles were less effective in stimulating an osteogenic response (Fig. 7C). In the longest recovery period group (8 h), sufficient time had passed to allow the cells to regain complete mechanosensitivity before the second and subsequent bouts were applied (Fig. 7B). Consequently, the amount of new bone formed per load cycle was much (>100 %) greater in the 8 h group. Although clearly suboptimal, the recovery data suggest that even shorter recovery periods (0.5–1 h) are more osteogenic than no recovery periods at all (0 h group).

Other investigators have noted the significance of a recovery phase for enhancing mechanically induced bone formation (Brand and Stanford, 1994). Chambers et al. (Chambers et al., 1993) reported that the trabecular bone formation rate in loaded rat tail vertebrae was more than five times higher in animals loaded for a total of 288 cycles over 8 days (36 cycles per day)

Table 2. *Effects of mechanical load cycle spacing (recovery period duration) on endocortical bone formation*

Time between cycles (s)	*N*	MAR (μm day^{-1}) Mean	MAR *P*-value[a]	MS/BS (%) Mean	MS/BS *P*-value	BFR/BS (μm^3 μm^{-2} year^{-1}) Mean	BFR/BS *P*-value
Bending							
0.5	9						
Right		2.44±0.63[b]	<0.01	23.46±1.87[b]	<0.001	217.61±52.03[b]	<0.01
Left		0.16±0.12		7.22±0.99		14.83±3.82	
3.5	8						
Right		1.64±0.32[b]	<0.01	19.20±2.94[b]	<0.01	131.40±30.66[b]	<0.01
Left		0.24±0.14		8.27±1.69		19.43±6.94	
7	9						
Right		2.17±0.43[b]	<0.01	21.50±3.51[b]	<0.01	196.56±51.81[b]	<0.05
Left		0.56±0.26		7.80±2.28		26.03±12.58	
14	8						
Right		2.74±0.45[b]	<0.01	38.89±3.05[b]	<0.001	375.29±52.19[b]	<0.001
Left		0.41±0.21		12.27±1.75		21.96±12.89	
Controls							
No loading	9						
Right		0.60±0.22	NS	6.51±1.11	NS	14.91±5.33	NS
Left		0.48±0.24		6.58±1.56		19.34±9.83	
0.5 sham	9						
Right		0.48±0.20	NS	10.82±1.69	NS	27.61±11.29	<0.05
Left		0.23±0.12		9.58±1.80		13.43±6.91	
14 sham	8						
Right		0.79±0.20	NS	12.15±2.54	NS	40.61±18.19	NS
Left		0.69±0.26		12.13±2.52		37.87±19.32	

Values are means ± S.E.M.

All experimental animals received 36 mechanical loading cycles per day. Force was applied in a haversine waveform at 2 Hz; peak force magnitude was 54 N.

[a]Probability associated with paired *t*-test between right and left values for each group. NS (not significant) indicates that the probability exceeds 0.05.

[b]Relative mean (calculated as right minus left) is significantly different from control group relative mean at $P<0.05$ (Dunnett's *post-hoc* test).

MAR, mineral apposition rate; MS, mineralizing surface; BS, bone surface; BFR, bone formation rate.

than in animals administered 360 of the same cycles all at once. Using the same model, Chow et al. (Chow et al., 1993) showed that rats loaded for a total of 270 cycles over 9 days (30 cycles per day) exhibited an approximately fourfold greater trabecular bone formation rate than rats that had been administered 300 cycles all at once. These studies show that rest periods lasting 24 h are important for mechanotransduction. Here, we show that only 8 h of recovery is necessary to restore mechanosensitivity to adapted bone cells.

Our second objective in this study was to determine the osteogenic effects of mechanical loading protocols that incorporate recovery periods, lasting only seconds, introduced between individual loading cycles. We found that loading schedules made up of 0.5 (back-to-back cycles), 3.5 and 7 s recovery periods resulted in approximately the same magnitude of osteogenic response. Allotting 14 s of recovery between cycles, however, resulted in significantly greater bone formation than was observed in any of the shorter recovery period groups. Unlike recovery between loading bouts (discussed above), recovery between loading cycles appears to operate according to a recovery threshold, somewhere between 7 and 14 s, rather than a dose response, which was observed in the long-term recovery animals.

Our short-term recovery results confirm in a rat model those reported previously (Srinivasan and Gross, 2000a) for the avian skeleton. They showed that adult turkey ulnae subjected to 100 bending cycles of a low-magnitude force exhibited an approximately sevenfold increase in labeled surface if the load cycles were separated by 10 s compared to ulnae administered back-to-back (2 Hz) cycles. Our data confirm their findings with regard to two points: (i) intercycle recovery periods in excess of 7 s are more osteogenic than no recovery periods (back-to-back cycles) and (ii) mineralizing surface is the bone formation variable in the adult skeleton most affected when short (>7 s) recovery periods are inserted between loading cycles. Similarly designed recovery experiments performed on the tibiae of growing mice have confirmed the osteogenic

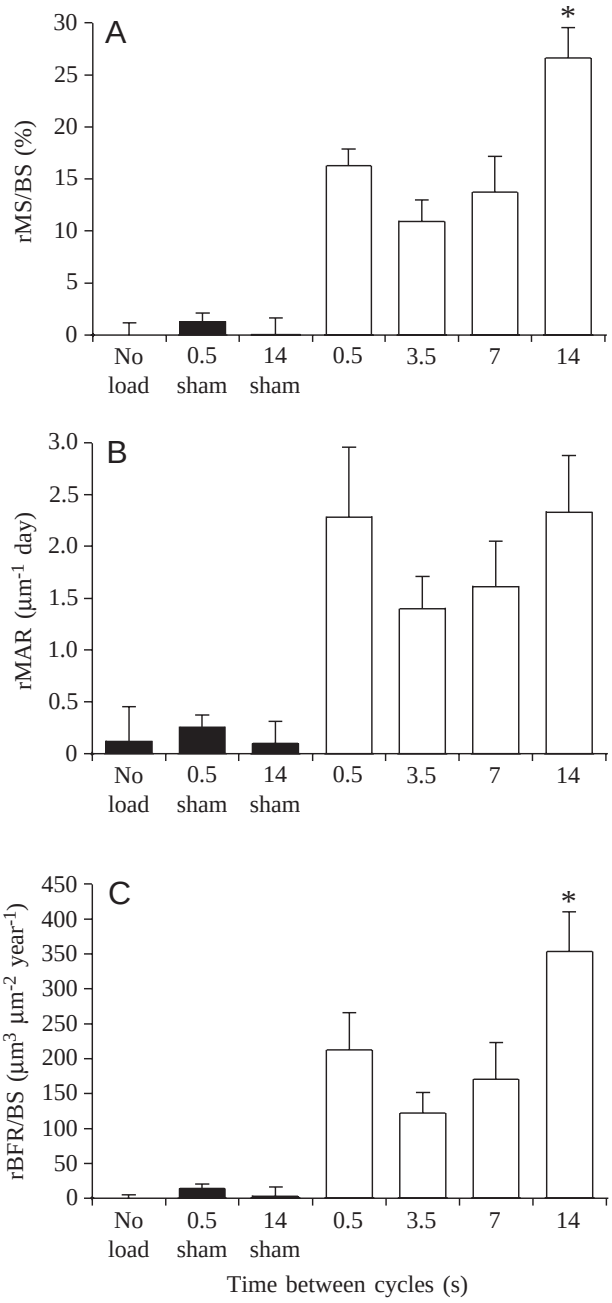

Fig. 6. (A) Relative mineralizing surface, (B) relative mineral apposition rates and (C) relative bone formation rate in the short-term recovery experiment. The relative (right minus left) mineralizing surface (rMS/BS), relative mineral apposition rate (rMAR) and relative bone formation rate (rBFR/BS) were enhanced in the bending groups (open columns) but not in the sham-bending or control groups (filled columns). The 14 s recovery group exhibited a significantly greater relative bone formation rate and relative mineralizing surface than the other three bending groups, which were not significantly different from one another, but were significantly different from each of the three controls. For comparison among bending groups, an asterisk indicates a significant difference from the 0.5, 3.5 and 7 s bending groups, based on Fisher's protected LSD at α=0.05. The significance of right (loaded) *versus* left (nonloaded) comparisons is indicated in Table 2. Values are means + S.E.M. For values of *N*, see Table 2.

potential of loading protocols that include short-term recovery periods (Srinivasan and Gross, 2001).

The dose response (long-term recovery) and threshold (short-term recovery) effects in relative bone formation rate observed among the bending groups resulted from changes in activated surface (rMS/BS) rather than from effects on appositional rate (rMAR). Thus, by inserting recovery periods (in both experiments), a greater percentage of the endocortical surface (rMS/BS) was covered with osteoblasts actively engaged in new bone formation. This suggests that the incorporation of recovery periods enhanced the recruitment and/or activation of osteoblasts along the bone surface. The lack of effect on rMAR suggests that the productivity of individual osteoblast teams at each new bone formation site was not affected by insertion of recovery periods.

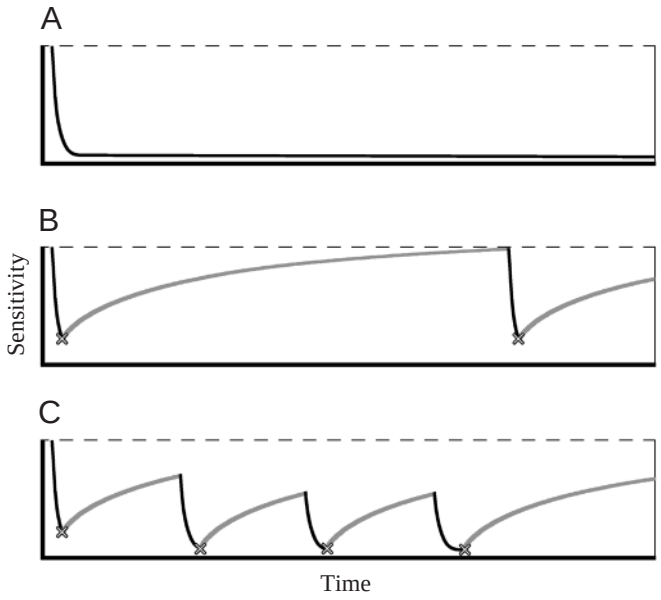

Fig. 7. (A) Bone cell mechanosensitivity declines soon after a loading bout (black line) is initiated, until a mechanosensory saturated state is reached where further mechanical stimulation produces no further osteogenic response. A recovery period, during which mechanical stimulation is drastically reduced or withheld, is required to return sensitivity to its preload value (100 % mechanosensitivity is represented by the broken line at the top). (B) When bone cells are stimulated, then allowed to recover (grey line) for a period sufficient to restore full sensitivity (approximately 8 h on the basis of the data presented) before the next bout is applied, the succeeding loading bout will stimulate fully sensitive cells. Consequently, a robust anabolic response can be generated from each bout. (C) The same initial loading bout as applied in B will stimulate the cells, but if subsequent bouts are initiated before full recovery has been achieved, the loading bouts will be applied to cells experiencing (temporary) mechanosensory impairment. Consequently, a compromised osteogenic response will result. Note that bouts 2–4 begin when the cells are at approximately 50–60 % of maximal sensitivity. B is representative of the 8 h recovery group; C is representative of the 0.5 h group.

The cellular mechanisms involved in mechanosensory loss and recovery are poorly understood. Cellular responses to mechanical loading involve a number of chemical and structural changes within the cell, some of which can occur in the time frame of milliseconds to seconds (e.g. Ca^{2+} channel activity), others in the time frame of minutes to hours (e.g. focal adhesion formation, cytoskeletal reorganization) and some occurring over several days (e.g. mitosis, apoptosis) (Banes et al., 1995). Desensitization/resensitization of bone cells might also occur on different biological levels, according to different time scales. We speculate that the loss and subsequent return of mechanosensitivity that occurs on a time scale of the order of hours, as demonstrated by the long-term recovery experiment, might be dependent upon the architecture of the actin cytoskeleton. *In vitro*, reorganization of the actin cytoskeleton of the osteoblast into stress fiber bundles is required for fluid shear-induced expression of genes linked to mechanotransduction and bone formation (Pavalko et al., 1998; Chen et al., 2000). Reorganization of the actin cytoskeleton results in an increase in whole-cell stiffness, which we speculate might make detection of mechanical signals less effective. If so, the cytoskeleton must be allowed time to disassemble before mechanosensitivity is restored. Glogauer et al. (Glogauer et al., 1997) reported that, in fibroblasts, stretch-activated cation-permeable channel activity, which is required in bone cells for mechanotransduction (Hung et al., 1996), is suppressed following mechanically induced F-actin accumulation at focal adhesions. Preliminary *in vitro* experiments conducted in our laboratory show that the reorganized cytoskeleton returns to its pre-stimulated morphology after approximately 8 h of post-stimulus recovery.

The cellular mechanisms involved in the short-term recovery phenomenon are also unclear, but they may involve ion recovery (e.g. repacking of Ca^{2+} into intracellular stores). Another possibility is that the short-term recovery phenomenon observed in our experiments is the result of a physical effect rather than a biological effect. Srinivasan and Gross (Srinivasan and Gross, 2000b) modeled extracellular fluid dynamics in the canalicular network of a dynamically loaded turkey ulna and reported that only 60 % (or less, depending on frequency) of the fluid velocity resulting from the first load cycle occurs in the second and subsequent cycles. Thus, the results from our short-term recovery experiment could be reflecting tissue viscoelasticity or fluid displacement effects rather than a time-dependent biological mechanism. If so, 14 s of recovery between cycles appears to be sufficient to restore the pre-load fluid distribution within the matrix. It is also possible that the long- and short-term recovery phenomena observed in our experiments could reflect opposite ends of the spectrum for the same biological mechanism. Elucidating the cellular mechanisms involved in bone cell mechanosensory saturation and resensitization holds great potential in maximizing the positive effects of mechanical loading, but further *in vitro* work is necessary to resolve these issues.

The short- and long-term recovery time constants that emerge from our experiments suggest that desensitization of bone cells to mechanical stimuli might be analogous to the well-characterized desensitization of G-protein-coupled receptors (GPRCs). Desensitization of GPRCs can occur *via* several different cellular mechanisms, each of which is associated with a distinct time constant. We detected desensitization and subsequent recovery events that occurred both in the 0–14 s range and in the 0.5–8 h range. The similarities between GPCR desensitization and mechanical desensitization might be a reflection of common origins: Reich et al. (Reich et al., 1997) showed that prostaglandin E_2 release from cultured osteoblasts was reduced by 85 % when cells were treated with the G-protein inhibitor GDPβS, which suggests a mediator role for G-proteins in bone cell mechanotransduction. However, a G-protein-coupled mechanoreceptor has yet to be identified in bone cells.

Several limitations of the experiments should be considered. First, only the endocortical surface of the tibial shaft was measured. In previous studies, we have found that the periosteal surfaces of tibiae exposed to bending exhibit a woven bone response similar in magnitude to those exposed to sham bending (Turner et al., 1994b; Robling et al., 2000); thus, we consider the periosteal measurements uninformative for studying a mechanically adaptive response. The endocortical surface, however, responds to bending but not sham bending, and the osteogenic response to bending is consistently lamellar in organization. Second, in the long-term recovery experiment, one of the sham groups did show a significant increase in rMS/BS in the loaded limb. However, inspection of Table 1 shows that the large rMS/BS value for the 8 h sham group is more the result of an unusually low MS/BS value in the left limb than of an elevated surface response in the sham-loaded limb. Third, a single load magnitude (54 N) and frequency waveform (2 Hz) were used. The time constants describing bone cell resensitization might change if the mechanical variables are manipulated.

In conclusion, recovery periods are important for returning desensitized cells to a sensitive state. Approximately 8 h of recovery between loading bouts can restore full mechanosensitivity to desensitized cells *in vivo*. Short-term recovery periods, introduced between loading cycles, are also important in enhancing the osteogenic response to loading; load cycles spaced 14 s apart result in a greater amount of bone formed per cycle than occurs when cycles are spaced by 7 s or less. Physical activity programs used as prophylaxis for bone loss might be met with greater success if appropriate recovery periods are structured into exercise routines. Selectively exposing the bone cell network to load cycles only when the network is highly mechanosensitive is a simple yet highly effective way to maximize the osteogenic effects of skeletal loading.

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