In Vivo Mechanical Loading Modulates Insulin-Like Growth Factor Binding Protein-2 Gene Expression in Rat Osteocytes

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Abstract. Mechanical stimulation is essential for maintaining skeletal integrity. Mechanosensitive osteocytes are important during the osteogenic response. The growth hormone-insulin-like growth factor (GH-IGF) axis plays a key role during regulation of bone formation and remodeling. Insulin-like growth factor binding proteins (IGFBPs) are able to modulate IGF activity. The aim of this study was to characterize the role of IGFBP-2 in the translation of mechanical stimuli into bone formation locally in rat tibiae. Female Wistar rats were assigned to three groups ($n = 5$): load, sham, and control. The four-point bending model was used to induce a single period of mechanical loading on the tibial shaft. The effect on IGFBP-2 mRNA expression 6 hours after stimulation was determined with nonradioactive *in situ* hybridization on decalcified tibial sections. Endogenous IGFBP-2 mRNA was expressed in trabecular and cortical osteoblasts, some trabecular and subendocortical osteocytes, intracortical endothelial cells of blood vessels, and periosteum. Megakaryocytes, macrophages, and myeloid cells also expressed IGFBP-2 mRNA. Loading and sham loading did not affect IGFBP-2 mRNA expression in osteoblasts, bone marrow cells, and chondrocytes. An increase of IGFBP-2 mRNA-positive osteocytes was shown in loaded (1.68-fold) and sham-loaded (1.35-fold) endocortical tibial shaft. In conclusion, 6 hours after a single loading session, the number of IGFBP-2 mRNA-expressing osteocytes at the endosteal side of the shaft and inner lamellae was increased in squeezed and bended tibiae. Mechanical stimulation modulates IGFBP-2 mRNA expression in endocortical osteocytes. We suggest that IGFBP-2 plays a role in the lamellar bone formation process.

Key words: Mechanical loading — Four-point bending system — Nonradioactive *in situ* hybridization — IGFBP-2 mRNA expression — *In vivo*

Mechanical stimulation is important for the maintenance of the skeleton of humans and other animals [1–9]. Bone serves as a structural support for the body

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and has the ability to modify its architecture throughout life. Mechanical stimulation regulates bone mass and architecture according to Frost's mechanostat hypothesis. Physical activity results in increased bone formation and decreased bone resorption, whereas inactivity has the opposite effect [10, 11].

The growth hormone-insulin-like growth factor (GH-IGF) axis plays an important role in the regulation of bone remodeling. Of the insulin-like growth factors (IGFs), IGF-I is predominantly expressed in rat bone and IGF-II is predominantly expressed in human bone [12–14]. IGFs are involved in bone formation after mechanical stimulation. Mechanical loading upregulates IGF-I mRNA expression in osteocytes, as shown in rat vertebra by Lean and colleagues using the caudal vertebra compression model [15]. Northern blot analysis also showed enhanced periosteal IGF-I mRNA expression after mechanical stimulation [16]. Besides an increase in IGF-I mRNA synthesis, Bravenboer and colleagues reported upregulation of IGF-I protein concentration in bone after mechanical stimulation using additional weight bearing in running rats (rat-with-backpack) [17].

As in other tissues, the autocrine and paracrine actions of IGFs in bone are modulated by a family of six structurally related binding proteins, the IGF binding proteins (IGFBPs). IGFBPs can be synthesized by osteoblasts [18, 19] and are capable of modulating the effects of IGFs by potentiating or inhibiting their action; some IGFBPs also have IGF-independent actions that can either inhibit or stimulate cellular function.

Since rat osteoblasts do not express IGFBP-1 [18, 20, 21], it is most likely that IGFBP-1 does not play a significant role during bone formation after mechanical stimulation in rats. Although IGFBP-2 is the major IGFBP synthesized by rat osteoblastic cells [18], a role for IGFBP-2 during mechanical loading has not been established yet. IGFBP-3 has both inhibitory and stimulatory effects on bone cells. IGFBP-4, which is locally produced by osteoblasts, has an inhibitory ef-

fect on bone metabolism, whereas locally synthesized IGFBP-5 by osteoblasts has a stimulatory effect [18, 22, 23]. However, the effects of IGFBP-4 on osteo-blasts are IGF-I-dependent, whereas IGFBP-5 proba-bly acts as a store for IGFs in bone tissue [18, 22, 23]. IGFBP-6 is expressed in rat osteoblasts [24]. In con-clusion, IGFBP-2 is the most abundant IGFBP in rat osteoblasts; however, the role of IGFBP-2 during bone formation after mechanical stimulation *in vivo* has not been determined yet. We hypothesized that IGFBP-2 mRNA expression level might be changed in bone cells during bone formation after mechanical stimulation.

The aim of this study, therefore, was to determine the localization of IGFBP-2 mRNA in the cortical tibial shaft after mechanical stimulation. To this end, we in-duced a single period of mechanical loading using the four-point bending model of Forwood and Turner [1, 2, 7]. This resulted in bone formation in the rat tibia 5−8 days after stimulation [25]. We developed an *in situ* hybridization method especially for bone tissue to detect the local osteogenic response at the cellular level 6 hours after a single period of dynamic loading.

Materials and Methods

Animals

Fifteen female 12-week-old Wistar rats (235 ± 12 g; Harlan, Zeist, The Netherlands) were randomly assigned to three weight-matched groups ($n = 5$/group): load, sham, and con-trol. The animal experiment was in accordance with the gov-ernmental guidelines for the care and use of laboratory animals and approved by the Institutional Animal Care and Use Committee of the VU University Medical Center (Amsterdam, The Netherlands).

In vivo Mechanical Loading

The right tibiae underwent "mediolateral" loading (load), sham loading (sham, in which the opposed pads were placed at the inner position, 11 mm apart), or no loading (control) using the four-point bending system of Forwood and Turner [1, 7]. Since loading will result in bending and squeezing of the tibia and sham loading only in squeezing of the tibia, the sham group was used as a control for the load group. The left tibiae served as contralateral controls. The four-point bending model [25] was used to generate a single period of dynamic loading of the right tibia in rats *in vivo* in order to detect acute changes of IGFBP-2 mRNA locally in bone tissue after stimulation by mechanical stress. The rats were subjected to a single episode of loading comprising 300 cycles (2 Hz) using a peak magnitude of 60 N. Forwood and Turner showed that a single loading session resulted in bone for-mation in the rat tibia 5−8 days after stimulation [25]. Characterization of the *in vivo* strain using the four-point bending system was reported by Akhter and colleagues [26]. Using the four-point bending system at our laboratory, we demonstrated bone formation at the endosteal surface of the rat tibia 15 days after a single loading session with a fre-quency of 2 Hz during 300 cycles and an applied peak magnitude of 60 N [27]. The loading experiment was per-formed under general anesthesia (2% isoflurane in 1 L/min O_2 and 2 L/min N_2O). The rats were killed exactly 6 hours after loading. This time point was based on the literature [15], which was confirmed by a time-course pilot experiment at our laboratory using real-time reverse-transcription polymerase chain reaction analysis (H. W. van Essen, personal commu-nication) showing the highest IGF-I mRNA expression 6 hours after loading. Since the actions of IGFs are influenced by their IGFBPs, we examined IGFBP-2 mRNA expression at 6 hours after loading. The tibiae were dissected and immediately fixed in 4% (w/v) paraformaldehyde (buffered in phosphate-buffered saline [PBS], pH 7.4) at 4°C for 24 hours.

Tissue

After fixation, the tibiae were decalcified in 10% ethylenedi-aminetetraacetic acid (EDTA) with 0.5% paraformaldehyde in PBS at 4°C for 4.5 weeks. Finally, the tibiae were washed in PBS and dehydrated through a series of ethanol and xylene at room temperature and embedded in paraffin.

As a positive control, brain tissue was used [28]. Brains were dissected rostrally to the cerebellum (interaural coordi-nate 0 mm) and the hippocampus (interaural coordinate 4 mm) in three coronal blocks and immediately fixed in 4% (w/v) paraformaldehyde (buffered in PBS, pH 7.4) at 4°C for 24 hours, followed by washing in PBS, dehydration through a series of ethanol and xylene at room temperature, and embedding in paraffin.

Reagents

All restriction enzymes and modifying enzymes were pur-chased from Roche Molecular Biochemicals (Mannheim, Germany), as well as digoxigenin-uridine triphosphate (UTP), antidigoxigenin Fab fragments, nitroblue tetrazolium chloride (NBT), 5-bromo-4-chloro-3-indolyl phosphate (BCIP), and blocking reagent. Nylon membranes were pur-chased from Qiagen (Hilden, Germany). Polyvinyl alcohol was obtained from Aldrich (Milwaukee, WI). Euparal mounting medium was purchased from Chroma Gesellschaft (Waldeck, Division Chroma, Münster, Germany). Silane-coated glass slides were obtained from Sigma-Aldrich (St. Louis, MO).

Mouse IGFBP-2 cDNA was kindly provided by Dr. S. L. S. Drop and Dr. J. W. van Neck (Department of Pediatrics, Sophia Children's Hospital, Erasmus University, Rotterdam, The Netherlands) via Dr. S. C. van Buul-Offers (Department of Metabolic and Endocrine Diseases, University Medical Center, Utrecht, The Netherlands).

Synthesis of Digoxigenin-Labeled Complementary RNA (cRNA) Probes

Standard *in vitro* transcription reactions were carried out using T7- and Sp6-RNA polymerase with digoxigenin-UTP as a substrate [29]. cDNA encoding mouse IGFBP-2 cDNA, cor-responding to amino acid position 98−258 and showing 95% homology with the corresponding rat IGFBP-2 cDNA frag-ment, was used as a template for the synthesis of antisense and sense digoxigenin-labeled RNA probe. The probe was specific for the mRNAs analyzed. The IGBP-2 probe did not show cross-reactivity with different types of mouse tissues (spleen, thymus, and brain) [28, 30, 31].

Nonradioactive In Situ Hybridization

Serial, longitudinal tibial sections (5 μm), which were cut in the posterior-anterior direction, and cross-sectional control brain sections (5 μm) were mounted onto RNase-free silane-coated glass slides and dried at 56°C for at least 3 days. Corre-sponding sections of the right and left tibia of one rat were mounted on the same glass slide. *In situ* hybridization was

performed on every fiftieth section with a total of five slides per tibia (i.e., sections I, II, II, IV, and V). All sections were dewaxed, rehydrated, and rinsed in water. The sections were pretreated with 0.2 N HCl for 15 minutes at room temperature, permeabilized in proteinase K (15 μg/mL) for 30 minutes at 37°C, and subjected to an acetylation treatment [32]. Sections were rinsed in 2× SSC (0.3 M sodium chloride and 0.03 M sodium citrate) and kept in this solution until the start of hybridization.

Hybridization was performed in a solution containing 50% formamide, 2× SSC, 1× Denhardt's solution, 250 μg/mL transfer RNA, 480 μg/mL herring sperm RNA, 10% dextransulfate, and the mouse IGFBP-2 digoxigenin-labeled cRNA probe at concentrations of 500 pg/μL (brain) and 1,000 pg/μL (tibia). Sections were hybridized overnight at 53°C. After hybridization, sections were washed with 50% formamide in 2× SSC at the hybridization temperature for 30 minutes and treated with RNase A (1 unit/mL) for 30 minutes at 37°C. Subsequently, sections were rinsed in 2× SSC, treated with 1% blocking reagent for 30 minutes, and incubated with sheep antidigoxigenin Fab fragments conjugated with alkaline phosphatase (1:1,500) overnight at 4°C.

Chromogenesis was performed in the dark with 0.38 mg/mL NBT and 0.19 mg/mL BCIP in the presence of 6% (w/v) polyvinyl alcohol [33], resulting in a blue precipitate. Finally, sections were counterstained with nuclear fast red, dehydrated through a series of ethanol, and mounted with Euparal. Sense probes were used to investigate the level of nonspecific binding.

Quantification and Statistics

First, a semiquantitative screening of all animals per group was performed on the antisense and sense slides to collect the *in situ* hybridization data. Sections I-V of all tibiae were scored in a semiquantitative manner, which was defined, on a Nikon Eclipse E800 microscope (Uvikon, Bunnik, The Netherlands), as follows: no expression (0), low expression (1), average expression (2), and high expression (3).

Effects of mechanical loading were expected in the shaft of the tibiae. Therefore, a second quantitative evaluation of the endocortical osteocytes expressing IGFBP-2 mRNA was performed with Lucia G Version 4.82 (Uvikon) using coded slides of sections II and III. The defined area of interest, which comprised the loading zone, started below the primary spongiosa at the endosteal side of the shaft following the total length of the shaft to the distal side (total length maximal 3,852 μm). At the endosteal side of the shaft, all osteocytes, which were positioned within 100 μm of the endosteal surface, were included in the area of interest. Within the area of interest, total osteocyte number and total IGFBP-2 mRNA-positive osteocyte number were measured.

The paired-samples Student's *t*-test and independent samples Student's *t*-test were used for statistical analysis using SPSS (Chicago, IL) version 9.0 for Windows. $P < 0.05$ was considered to reflect statistical significance.

Photography

Brightfield photographs with different magnifications (objectives ×10, ×20, and ×40) were made using a Leica microscope (DM4000B) with a digital camera (Leica DC500) and Leica software IM50 (Leica Microsystems, Rijswijk, The Netherlands).

Results

Endogenous IGFBP-2 mRNA Expression in Tissue

The control brain showed IGFBP-2 mRNA expression in the choroid plexus (Fig. 1A) and the neurons of the medulla oblongata (data not shown). In the internal control, i.e., the growth plate, IGFBP-2 mRNA was located in chondrocytes of the proliferative and hypertrophic zone (Fig. 1C). In control tibiae, IGFBP-2 mRNA was expressed in osteoblasts, which were situated against the surface of trabecular bone (Fig. 1C, E) and the endosteal side of the shaft (Fig. 1G). IGFBP-2 mRNA expression was also observed in some trabecular osteocytes and in cortical osteocytes, which were situated within the first lamella at the endosteal side of the shaft (Fig. 1G). The endocortical osteocytes, which were located within the deeper lamellae (Fig. 1G), and the periosteal osteocytes (Fig. 1D) did not express IGFBP-2 mRNA. IGFBP-2 mRNA was also expressed in the intracortical endothelial cells of blood vessels (Fig. 1G) and in the periosteum of control tibiae (Fig. 1D). Some cells of the bone marrow, i.e., megakaryocytes, macrophages, and myeloid cells, also expressed IGFBP-2 mRNA (Fig. 1E). No differences of IGFBP-2 mRNA expression between the right and left tibiae of the control group were observed using semiquantitative analysis.

Control hybridizations with the corresponding sense RNA probe exhibited no signals in the brain (Fig. 1B) and tibia (Fig. 1F).

Effect of Mechanical Loading on IGFBP-2 mRNA Expression in Osteocytes of the Tibial Shaft

Induced IGFBP-2 mRNA expression was observed in osteocytes within the first lamella and within multiple layers at the endosteal side of the shaft of loaded tibia (1.68-fold increase, $n = 5$) (Fig. 1J) and sham-loaded tibia (1.35-fold increase, $n = 5$) (Fig. 1H) in contrast to the control tibia ($n = 10$), where IGFBP-2 mRNA expression was only seen within the first lamella of the endosteal side of the shaft (Fig. 1G).

The proportion of IGFBP-2 mRNA-positive osteocytes was 25.5 ± 12.6% (mean ± standard deviation [SD], $n = 5$) for loaded tibiae and 17.2 ± 10.7% (mean ± SD, $n = 5$) for the contralateral control tibiae (Figs. 1G, J and 2A). Sham-loaded tibiae and the contralateral control tibiae showed a proportion of IGFBP-2 mRNA-positive osteocytes of 20.5 ± 7.5% (mean ± SD, $n = 5$) and 13.2 ± 4.2% (mean ± SD, $n = 5$), respectively (Figs. 1H and 2B). Mechanical loading significantly increased the number of IGFBP-2 mRNA-synthesizing osteocytes in loaded tibiae ($P = 0.001$) and in sham-loaded tibiae ($P = 0.031$) (Fig. 2A, B). No statistical differences of IGFBP-2 mRNA-positive osteocytes were observed between the contralateral controls of the loaded and sham-loaded tibiae ($P = 0.475$) or between the loaded and sham-loaded tibiae ($P = 0.468$).
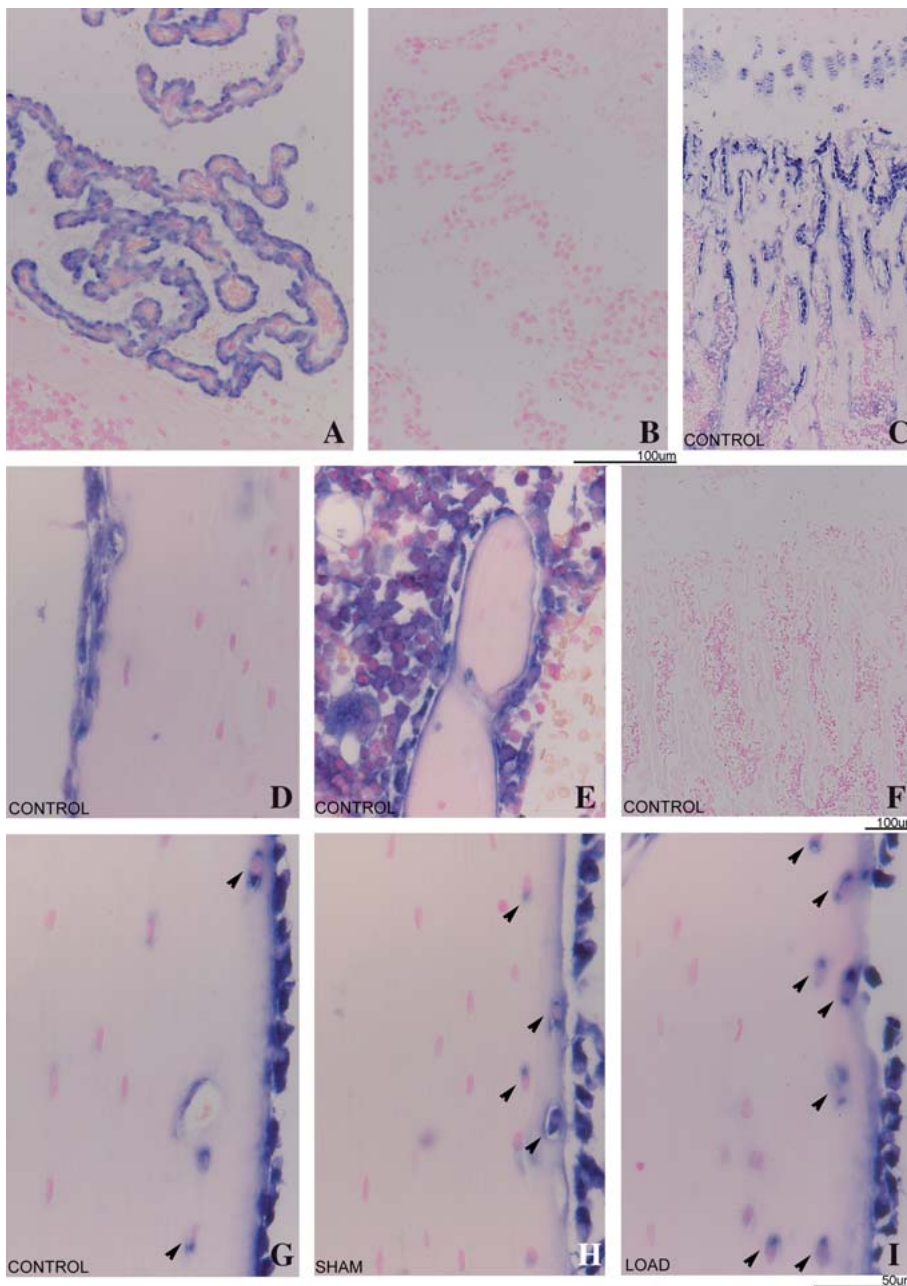
Fig. 1. Expression of IGFBP-2 mRNA in rat brain sections (**A, B**) and in longitudinal control (**C-G**), sham-loaded (**H**), and loaded (**J**) tibial sections of 12-week-old female Wistar rats. The RNA signal is shown as a blue precipitate. The sections were counterstained with nuclear fast red and visualized under brightfield illumination. The scale bars represent 100 μm (**A, B**), 50 μm (**D, E, G-J**), and 100 μm (**C, F**). (**A**) IGFBP-2 mRNA expression within the choroid plexus of a control rat brain; red blood cells are negative and do not express IGFBP-2 mRNA. (**B**) Hybridization with representative IGFBP-2 sense probe showed no signal in the choroid plexus. (**C**) IGFBP-2 mRNA expression in the chondrocytes of the growth plate and osteoblasts of the trabecular bone. (**D**) IGFBP-2 mRNA expression in the periosteum of a control tibia; the osteocytes in the periosteal side of the shaft do not express IGFBP-2 mRNA. (**E**) IGFBP-2 mRNA expression in osteoblasts situated against the surface of normal trabecular bone and IGFBP-2-positive bone marrow cells. (**F**) Hybridization with a corresponding IGFBP-2 sense probe showed no signal in the osteoblasts, chondrocytes, and bone marrow cells of a control tibia. (**G**) IGFBP-2 mRNA expression within the superficial endocortical osteocytes (indicated by *arrowheads*), osteoblasts, and intracortical endothelial cells of a blood vessel of a control tibia. (**H**) IGFBP-2 mRNA expression in the endocortical osteocytes within multiple layers (indicated by *arrowheads*) and osteoblasts of a sham-loaded tibia. (**J**) IGFBP-2 mRNA expression in the endocortical osteocytes within multiple layers (indicated by *arrowheads*) and osteoblasts of a loaded tibia.
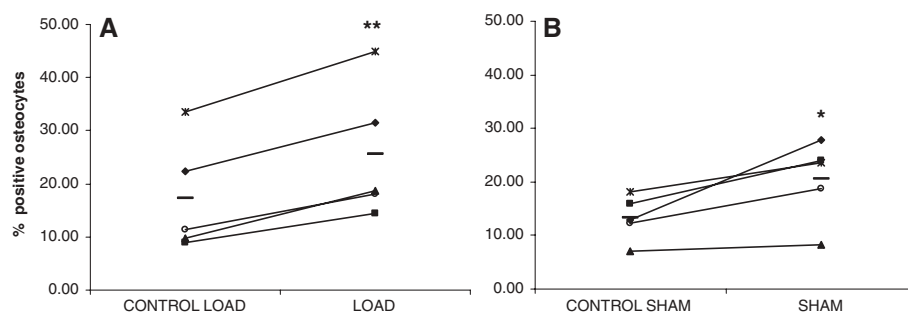
Fig. 2. Influence of mechanical loading on IGFBP-2 mRNA expression in osteocytes at the endosteal side of the shaft within individual rat tibiae. Values are expressed as (**A**) percentage IGFBP-2 mRNA-positive osteocytes in control versus contralateral loaded tibiae ($^{**}P = 0.001$, $n = 5$) and (**B**) percentage IGFBP-2 mRNA positive osteocytes in control versus contralateral sham-loaded tibiae ($^{*}P = 0.031$, $n = 5$). No statistical differences of IGFBP-2 mRNA-positive osteocytes were observed between the control load and control sham groups ($P = 0.475$) or between the load and sham groups ($P = 0.468$). The lines connect the left and right tibiae of the same rat. The mean values of each group are represented by a bar.

Effects of Mechanical Loading on IGFBP-2 mRNA Expression in Osteoblasts, Chondrocytes, and Bone Marrow Cells

No differences in IGFBP-2 mRNA expression between loaded, sham-loaded, and control tibiae were observed in the osteoblasts, chondrocytes, and bone marrow cells by semiquantitative screening. No differences in morphology of the cells were observed between groups.

Discussion

This *in vivo* study showed an increase in the number of endocortical osteocytes expressing IGFBP-2 mRNA in loaded (1.68-fold) and sham-loaded (1.35-fold) tibiae 6 hours after a single period of mechanical loading with the four-point bending system. The upregulation of IGFBP-2 mRNA expression was restricted to osteocytes at the endosteal side of the shaft and multiple inner lamellae. No difference between IGFBP-2 mRNA expression in the left and right control tibia was observed. We conclude that bending and squeezing of rat tibiae result in an increase of locally synthesized IGFBP-2 mRNA by endocortical osteocytes. We suggest that IGFBP-2 plays an as yet unidentified role in the lamellar bone formation process.

We suggest that the IGFBP-2-producing osteocytes of the loaded tibiae are involved in the increased lamellar bone formation after mechanical loading. Forwood and colleagues reported that a single short period of loading using the four-point bending system resulted in an increased lamellar bone formation rate at the endosteal surface of rat tibia [25]. This has been confirmed in a validation study at our laboratory [27]. The lamellar bone formation is located at the endosteal side of the shaft, which is identical to the location of newly synthesized IGFBP-2 mRNA after mechanical loading, as demonstrated in this study. IGFBP-2 mRNA expression was restricted to this area and was not ob-

served in the osteocytes at the periosteal side of the shaft. Mechanical loading with an external bending load of 60 N *in vivo* will result in an increased woven bone formation rate at the periosteal surface as a result of irritation of the periosteum and increased lamellar bone formation rate at the endosteal surface [1]. We suggest that IGFBP-2 mRNA may be less involved in woven bone formation because the osteocytes at the periosteal side of the shaft did not express IGFBP-2. This suggests that IGFBP-2 mRNA in the mechanosensitive osteocytes is specifically important for lamellar bone formation.

In addition, this study showed that the sham-loaded tibiae have increased IGFBP-2 mRNA production in endocortical osteocytes 6 hours after sham loading. Apparently, besides bending, squeezing alone is also responsible for the upregulation of IGFBP-2 mRNA in the endocortical osteocytes. However, squeezing did not induce lamellar bone formation.

Remarkably, there is a large range in the data for the percentage of IGFBP-2 mRNA-positive osteocytes in the contralateral control tibiae of the load group in comparison to the data of the contralateral tibiae of the sham group. This could be caused by the biological variation between the individuals because the animals were randomly divided between the groups and there is no significant statistical difference between those tibiae.

For this study we used the four-point bending model of Forwood because this apparatus produces a controlled mechanical strain in the tibia of living rats. An advantage of this approach is that it does not require surgical intervention and allows normal physical activity after the loading session [1, 7]. The osteogenic response will occur at a distinct location in bone. Therefore, we used the *in situ* hybridization technique in order to detect the local osteogenic response at the cellular level. Nonradioactive *in situ* hybridization is a powerful and sensitive technique to localize gene expression within

decalcified rat tibiae. It was demonstrated that the IG-FBP-2 probe is specific because IGFBP-2 mRNA expression is present in the choroid plexus and the neurons of the medulla oblongata, as described earlier [28], and in the chondrocytes of the proliferative and hypertrophic zone of the growth plate [34].

Nevertheless, this study has some limitations. The applied load of 60 N is supraphysiological, and 6 hours after a single loading session is too early to demonstrate bone formation.

In summary, this study shows an increase of the number of osteocytes at the endosteal side of the shaft and inner lamellae expressing IGFBP-2 mRNA 6 hours after mechanical loading and sham loading *in vivo*. We conclude that these IGFBP-2-producing osteocytes in the endosteal shaft are mechanosensitive. We suggest that IGFBP-2 plays a role in the lamellar bone formation process.

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