Effect of mechanical loading on insulin-like growth factor-I gene expression in rat tibia

Christianne M A Reijnders[1], Nathalie Bravenboer[1,2], Annechien M Tromp[1], Marinus A Blankenstein[2] and Paul Lips[1]

Departments of [1]Endocrinology and [2]Clinical Chemistry, VU University Medical Center, De Boelelaan 1117, brug 124, PO Box 7057, 1081 HV Amsterdam, The Netherlands

(Requests for offprints should be addressed to N Bravenboer; Email: n.bravenboer@vumc.nl)

Abstract

Mechanical loading plays an essential role in maintaining skeletal integrity. Mechanical stimulation leads to increased bone formation. However, the cellular and molecular mechanisms that are involved in the translation of mechanical stimuli into bone formation, are not completely understood. Growth factors and osteocytes, which act as mechanosensors, play a key role during the bone formation after mechanical stimulation. The aim of this study was to characterize the role of IGF-I in the translation of mechanical stimuli into bone formation locally in rat tibiae. Fifteen female Wistar rats were randomly assigned to three groups ($n=5$): load, sham-loaded, and control. The four-point bending model of Forwood and Turner was used to induce a single period of mechanical loading on the tibia shaft. The effects of mechanical loading on IGF-I mRNA expression were determined with non-radioactive *in situ* hybridization on decalcified tibiae sections, 6 h after the loading session. Endogenous IGF-I mRNA was expressed in trabecular and cortical osteoblasts, some trabecular and sub-endocortical osteocytes, intracortical endothelial cells of blood vessels, and periosteum. Megakaryocytes, macrophages, and myeloid cells also expressed IGF-I mRNA. In the growth plate, IGF-I mRNA was located in proliferative and hypertrophic chondrocytes. Mechanical loading did not affect the IGF-I mRNA expression in osteoblasts, bone marrow cells, and chondrocytes, but the osteocytes at the endosteal side of the shaft showed a twofold increase of IGF-I mRNA expression. The proportion of IGF-I mRNA positive osteocytes in loaded tibiae was $29{\cdot}3 \pm 12{\cdot}9\%$ (mean $\pm$ S.D.; $n=5$), whereas sham-loaded and contra-lateral control tibiae exhibited $16{\cdot}7 \pm 4{\cdot}4\%$ ($n=5$) and $14{\cdot}7 \pm 4{\cdot}2\%$ ($n=10$) respectively ($P<0{\cdot}05$). Lamellar bone formation after a single mechanical loading session was observed at the endosteal side of the shaft. In conclusion, a single loading session results in a twofold up-regulation of IGF-I mRNA synthesis in osteocytes which are present in multiple layers extending into the cortical bone of mechanically stimulated tibia shaft 6 h after loading. This supports the hypothesis that IGF-I, which is located in osteocytes, is involved in the translation of mechanical stimuli into bone formation.

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Introduction

Mechanical loading plays an essential role in maintaining the skeletal integrity in both humans and animals (Forwood & Parker 1991, Turner *et al.* 1991, 1994, Smith & Rutherford 1993, Yeh *et al.* 1993, Hamdy *et al.* 1994, Torrance *et al.* 1994, van der Wiel *et al.* 1995, Forwood *et al.* 1998). Bone modifies its structure throughout life in order to serve as a structural support for the body. According to the mechanostat theory, decreased physical activity results in decreased bone formation and increased bone resorption, whereas increased physical activity has the opposite effect (Yeh *et al.* 1993). It has been suggested that osteocytes function as mechanosensors via canalicular processes and communicating gap junctions in the early stage of bone remodeling (Cowin *et al.* 1991, Nomura & Takano-Yamamoto 2000).

Several *in vivo* studies have shown that many biochemical signal molecules are involved in the translation of mechanical stimuli into bone formation including glucose-6-phosphate dehydrogenase (G6PD) (Skerry *et al.* 1989, Lanyon 1992), c-fos (Raab-Cullen *et al.* 1994, Lean *et al.* 1996), cAMP (Davidovitch *et al.* 1984), cyclooxygenase (COX-2) (Forwood 1996), nitric oxide (NO) and prostanoid (Pitsillides *et al.* 1995), insulin-like growth factors (IGFs) (Raab-Cullen *et al.* 1994, Lean *et al.* 1995, Bravenboer *et al.* 2001), transforming growth factor-β (TGF-β) (Raab-Cullen *et al.* 1994, Bravenboer *et al.* 2001), protein kinase B (PKB or Akt) (Skerry & Suva 2003), and glutamate transporter (GLAST) (Mason *et al.* 1997, Skerry 1999, Skerry & Genever 2001, Skerry & Suva 2003). These responses to *in vivo* mechanical loading are time- and spatially-dependent. Early strain related changes within 5 min after loading are shown in osteocytes in which the G6PD activity is increased (Skerry *et al.*

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1989, Lanyon 1992), whereas an increase of IGF-I mRNA expression is located on trabecular surfaces and in osteocytes of the diaphysial cortex (cortical and trabecular osteocytes) of rat caudal vertebrae within 6 h after a single loading session (Lean *et al.* 1995).

The aim of this study was to characterize the role of IGF-I mRNA in the cortical tibia shaft during the translation of mechanical stimuli into bone formation. To this end, we developed an *in situ* hybridization method especially for bone tissue to detect the local osteogenic response on cellular level 6 h after a single period of dynamic loading. To induce a single period of mechanical loading the four-point bending model of Forwood and Turner has been used (Turner *et al.* 1991, 1994, Forwood *et al.* 1998) resulting in bone formation in the rat tibia 5–8 days after stimulation (Forwood *et al.* 1996).

Materials and Methods

Study design

This study comprised two parts: (1) validation study of the four-point bending system to verify bone formation after a single mechanical loading session and (2) detection of changes in mRNA expression of IGF-I after a single mechanical loading session with non-radioactive *in situ* hybridization.

The animal experiments were in accordance with the governmental guidelines for care and use of laboratory animals and approved by the Institutional Animal Care and Use Committee (IACUC) of the VU University Medical Center Amsterdam, The Netherlands.

Validation study four-point bending system

Female Wistar rats of 12-weeks old (Harlan, Zeist, The Netherlands) were randomly assigned into two groups ($n=3$ per group): LOAD and SHAM. The right tibiae underwent 'medio-lateral' loading (LOAD) (distance between the centers of the loading pads: upper pads, 11 mm and lower pads, 23 mm) or sham-loading (SHAM) (opposed pads were placed at the inner position: 11 mm) using the four-point bending system of Forwood and Turner (Fig. 1, Turner *et al.* 1991, Forwood *et al.* 1998). 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 control for the LOAD group. The left non-loaded tibiae served as contra-lateral controls (CONTROL). The four-point bending model (Forwood *et al.* 1996) was used to generate a single period of dynamic loading of the right tibia in rats *in vivo*. The rats were subjected to a single episode of loading comprising 300 cycles (2 Hz) using a peak magnitude of 60 N, which generates a mean strain of 2664 μstrain in the loaded tibia compared with a mean strain of 350 μstrain in the sham-loaded tibia (Forwood *et al.* 1998). Tetracycline (25 mg/kg body weight) was administered intraperitoneally to the rats, 5 and 12 days after the single loading session. The rats were killed 15 days after loading.

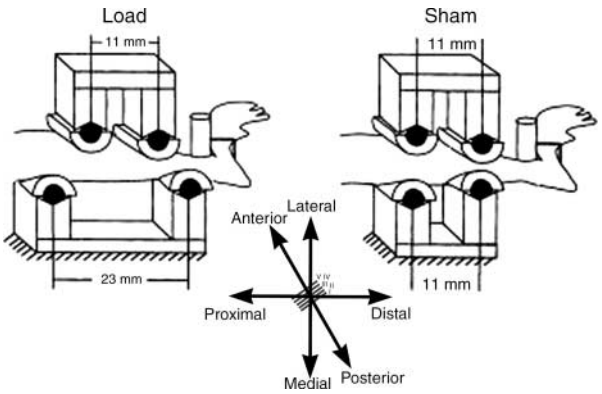

Figure 1 Four-point bending model including a schematic of sectioning. LOAD, a mediolateral bending moment is produced in the shaft of the tibia when a force is applied to the upper device. The distance between the two upper padded load points is 11 mm and between the lower points is 23 mm; SHAM, the lower points are removed inward so that they directly oppose the upper points (11 mm). When a force is applied to the upper device, it will squeeze the shaft of the tibia and the surrounding soft tissues, like muscles, but no bending of the shaft occurs. The direction of sectioning was performed in the posterior–anterior direction. The semi-quantitative analysis comprised of 5 sections per tibia with a distance of 50 sections in between respectively shown as sections I–V, covering the whole tibia. Figure used with written permission of Dr CH Turner. Reprinted from Robling AG, Burr DB & Turner CH 2001 Skeletal loading in animals. *Journal of Musculoskeletal and Neuronal Interactions* **1** 249–262.

The tibiae were removed, fixed in 4% phosphate buffered formaldehyde, and embedded in methyl metacrylate (mma). Three cross-sectional sections of 30 μm were sawn with a sawing microtome (FMTA, Amsterdam, The Netherlands) through the region of applied loading or through the corresponding region of the contra-lateral tibia. The endosteal surfaces of all sections were used for histomorphometry measurements. Mineralizing surface (MS/BS (% BS: bone surface)) and mineral apposition rate (MAR (μm/day)) were measured semi-automatically using Osteomeasure (OsteoMetrics Inc Decatur, GA, USA). MAR was defined as the mean distance between the fluorescent labels, divided by the labeling period. Nomenclature and calculations were according to the American Society of Bone and Mineral Research nomenclature committee. Data are expressed as mean values of the analyzed sections. Since the sample size was limited, statistical analyses were not made.

In vivo *mechanical loading* in situ *hybridization*

Fifteen female 12-week-old Wistar rats (235 ± 12 g) (Harlan) were randomly assigned to three weight-matched groups ($n=5$/group): LOAD, SHAM, and CONTROL. The right tibiae underwent 'medio-lateral' loading (LOAD), sham-loading (SHAM), or no loading (CONTROL) using the four-point bending system of Forwood and Turner (Fig. 1, Turner *et al.* 1991, Forwood *et al.* 1998). The left tibiae served as contra-lateral controls. The four-point bending model (Forwood

et al. 1996) 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 IGF-I 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. The loading experiment was performed under general anesthesia (2% isoflurane in 1 l/min O_2 and 2 l/min N_2O). The rats were killed exactly 6 h after loading. This time point was based on literature (Lean *et al.* 1995) which was confirmed by a time-course pilot experiment at our laboratory using real-time reverse transcriptase (RT)-PCR analysis (H W van Essen, personal communication). The tibiae were dissected and immediately fixed in 4% (wt/vol) paraformaldehyde (buffered in PBS, pH 7·4) at 4 °C for 24 h.

Tissue

After the fixation, the tibiae were decalcified in 10% EDTA with 0·5% paraformaldehyde in PBS at 4 °C for 4½ weeks. Finally, the tibiae were washed in PBS and dehydrated through a series of ethanol and xylene at room temperature and embedded in paraffin.

Control brains were dissected rostrally to the cerebellum (interaural coordinate 0 mm) and the hippocampus (interaural coordinate 4 mm) in three coronal blocks and immediately fixed in 4% (wt/vol) paraformaldehyde (buffered in PBS, pH 7·4) at 4 °C for 24 h, 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 purchased from Roche Molecular Biochemicals, as well as digoxigenin-UTP, anti-digoxigenin Fab fragments, nitro-blue-tetrazolium chloride, 5-bromo-4-chloro-3-indolyl phosphate, and blocking reagent. Nylon membranes were purchased from Qiagen. Polyvinyl alcohol was obtained from Aldrich (Milwaukee, WI, USA). Euparal mounting medium was purchased from Chroma Gesellschaft (Schmid GmbH, Köngen, The Netherlands). Silane-coated glass slides were obtained from Sigma-Aldrich (St Louis, MO, USA).

rRNA and human IGF-I cDNA was kindly provided by Dr S C van Buul-Offers (Department of Metabolic and Endocrine Diseases, University Medical Center Utrecht, 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 (Melton *et al.* 1984). cDNA encoding ribosomal 28S RNA and human IGF-I (259 bp, containing exon 2 and 3, 120–379 nt, gene ID X00173) (Jansen *et al.* 1983) were used as a template for the synthesis of antisense and sense digoxigenin-labeled RNA probe. The probe was specific for the mRNAs analyzed. The IGF-I probe was checked for cross-hybridization using *in situ* hybridization on spleen and growth plate cartilage (Smink *et al.* 2002).

Non-radioactive in situ *hybridization*

Serial, longitudinal, tibia sections (5 μm), which were cut in 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. *In situ* hybridization was performed on every 50th section with a total of five slides per tibia (i.e., sections I, II, III, IV, and V; Fig. 1). Sections I, II, III, IV, and V included the loading zone of the loaded and sham-loaded tibia and were taken to obtain regular sampling throughout the whole tibia. Corresponding sections of the right and the left tibia sections of one rat were mounted on the same glass slide. All sections were dewaxed, rehydrated, and rinsed in water. The sections were pretreated with 0·2 M HCl for 15 min at room temperature, permeabilized in proteinase K (15 μg/ml) for 30 min at 37 °C, and subjected to an acetylation treatment (Wilkinson 1992). The 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 the hybridization.

Hybridization was performed in a solution containing 50% formamide, 2×SSC, 1×Denhardt's solution, 250 μg/ml tRNA, 480 μg/ml herring sperm RNA, 10% dextran-sulfate and the rRNA digoxigenin-labeled cRNA probe at a concentration of 250 pg/μl and the human IGF-I (hIGF-I) digoxigenin-labeled cRNA probe at a concentration of 1500 pg/μl. Sections were hybridized overnight at 53 °C. After hybridization, sections were washed with 50% formamide in 2×SSC at the hybridization temperature for 30 min and treated with RNase A (1 unit/ml) for 30 min at 37 °C. Subsequently, sections were rinsed in 2×SSC, treated with 1% blocking reagent for 30 min, and incubated with sheep anti-digoxigenin Fab fragments conjugated with alkaline phosphatase (1:1500) O/N at 4 °C.

Chromogenesis was performed in the dark with 0·38 mg/ml nitro-blue-tetrazolium chloride (NBT) and 0·19 mg/ml 5-bromo-4-chloro-3-indolyl phosphate (BCIP) in the presence of 6% (wt/vol) polyvinyl alcohol (De Block & Debrouwer 1993) resulting in a blue precipitate. The sections were counterstained with nuclear fast red. Thus, positive cells will have a blue cytoplasmatic staining, whereas negative cells will be pink. Finally, the sections were dehydrated through a series of ethanol and mounted with Euparal (Waldeck Gmbh & Co Division Chroma, Münster, Germany). Sense probes were used to investigate the level of non-specific binding.

Quantification and statistics

First, a semi-quantitative screening of five different animals per group was performed to collect the *in situ* hybridization data. All tibiae were scored in a semi-quantitative manner

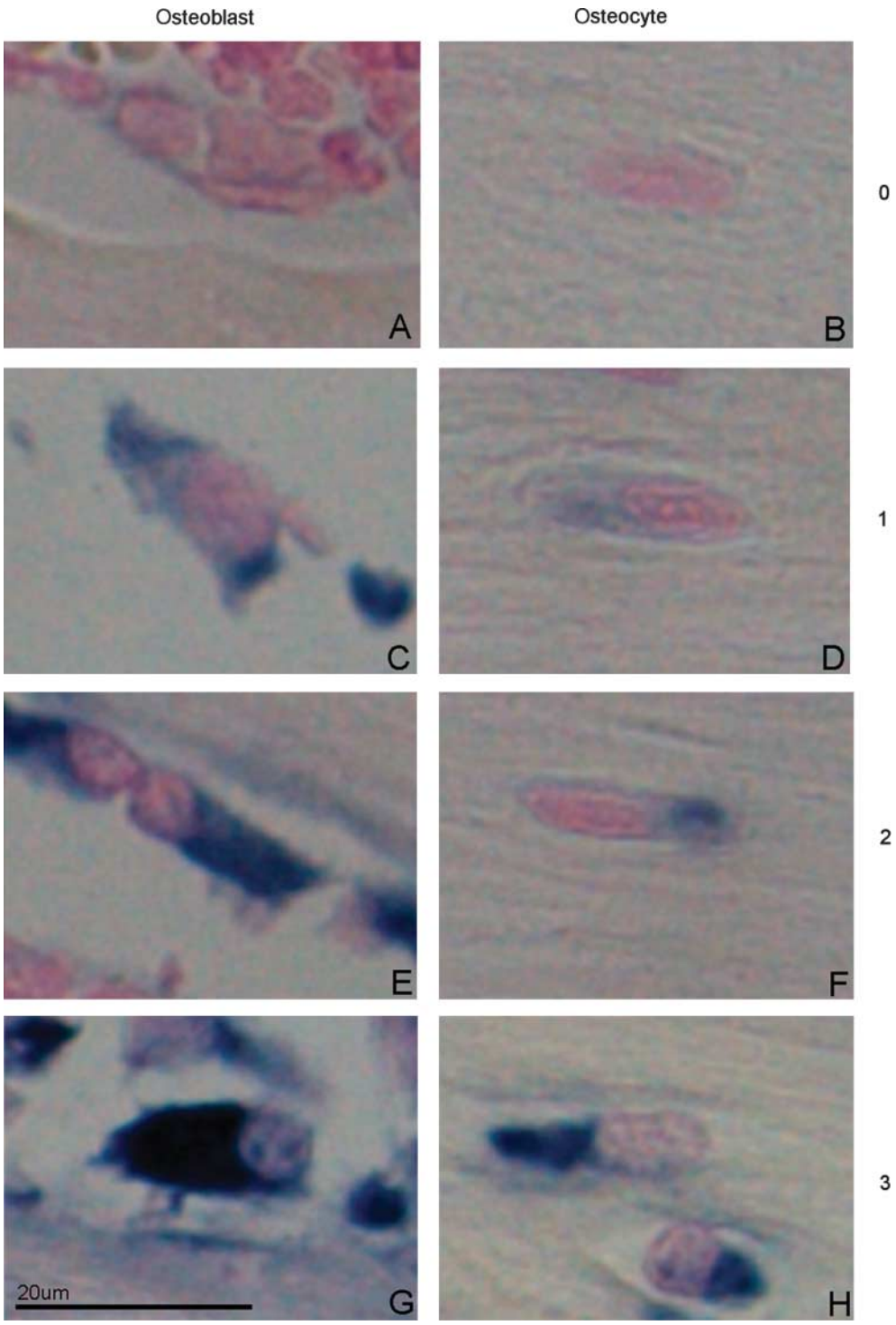

Figure 2 Representation of the semi-quantative scoring system assessing the levels of expression in the osteoblasts (A, C, E, and G) and the osteocytes (B, D, F, and H) in the tibiae of a 12-week-old female Wistar rat. Level 0, no expression (A and B); level 1, low expression (C and D); level 2, average expression (E and F); and level 3, high expression (G and H). The scale bar represents 20 μm (A–H).

which was defined as follows: no expression (0), low expression (1), average expression, (2) and high expression (3) as shown in Fig. 2.

Effects of mechanical loading were expected in the shaft of the tibiae. Therefore, quantitative scoring of the endocortical osteocytes expressing IGF-I mRNA was performed with Lucia G Version 4.82 (Uvikon, Bunnik, The Netherlands) 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 3852 μm). At the endosteal side of the shaft all osteocytes, which were positioned within 100 μm endosteal surface, were included in the area of interest. Within the area of interest, total osteocyte number and total IGF-I mRNA positive osteocyte number were measured using coded slides, resulting in a percentage of IGF-I mRNA positive osteocytes. We analyzed the sections of all rats per group within two different *in situ* hybridizations. The intra-individual mean S.D. of the quantitative measurement of the percentage positive osteocytes is 2·00% and the inter-individual mean S.D. is 0·94%.

ANOVA (one-way ANOVA) was used for statistical analysis using SPSS version 9.0 for Windows. A P value of $<0{\cdot}05$ was considered to reflect statistical significance.

Photography

Brightfield photographs were made using Leica microscope (DM4000B) with a digital camera (Leica DC500) and the Leica software program IM50 (Leica Microsystems, Rijswijk, The Netherlands).

IGF-I protein assay

IGF-I protein was measured in serum by a RIA for the quantitative measurement of human somatomedin-C (Bio-Source, International, Camarillo, CA, USA). The intra-assay coefficient of variation was $<5{\cdot}2\%$ ($n=15$) for levels between 925 and 2000 ng/ml and the inter-assay coefficient of variation was $<10\%$ ($n=20$) for levels between 120 and 500 ng/ml. The minimal detectable concentration was 35 ng/ml.

Results

Validation study four-point bending system

The loaded right tibiae of the rats showed lamellar bone formation at the endosteal surface (Fig. 3A–C) and woven bone formation at the periosteal surface (Fig. 3A). The MS/BS expressed as a percentage of the bone surface of the loaded tibiae was 58%, whereas the contralateral control tibiae showed a MS/BS of 40%. The MS/BS of sham-loaded tibiae and its contralateral control tibiae was for both 38% (Table 1).

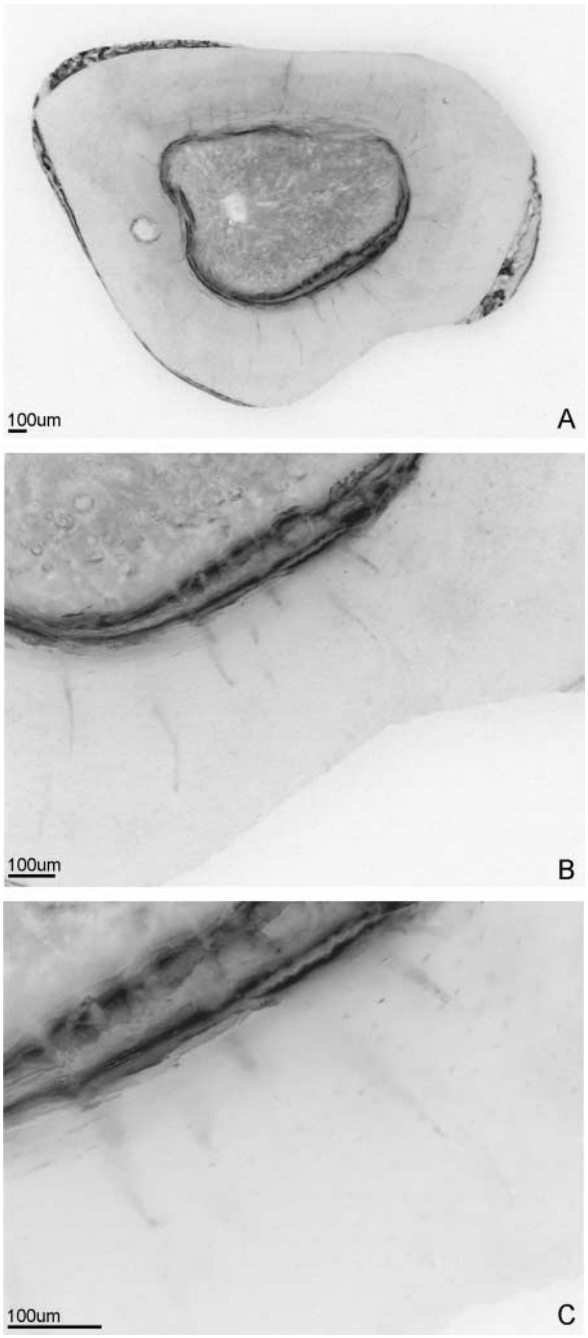

Figure 3 Photographs of fluorescence label in a cross-sectional sawing section of a loaded tibia. At the endosteal bone surface lamellar bone formation is shown (double label; A–C), whereas at the periosteal bone surface woven bone formation is present (A).

IGF-I protein concentrations in serum

The IGF-I protein concentrations in serum, expressed as mean ± S.E.M., were 1506 ± 126 ng/ml in the control group, 1409 ± 155 ng/ml in the load group, and 1408 ± 93 ng/ml in

Table 1 Mineralizing surface and mineral apposition rate of the endosteal bone surface. Data are mean values for each group of rats.

	Right tibiae	**MS/BS** (%)	**MAR** (μm/day)	**Left tibiae**	**MS/BS** (%)	**MAR** (μm/day)
n						
3	Load	58	1·41	Control	40	1·71
3	Sham	38	1·52	Control	38	1·58

MS/BS, mineralizing surface is expressed as a percentage of the bone surface; MAR, mineral apposition rate. MAR is defined as the mean distance between the fluorescent labels divided by the labeling period.

the sham group respectively. Differences between groups were not observed (P value = 0·808).

Ribosomal 28S RNA expression in tibiae

Analysis of ribosomal 28S RNA in control tibiae using non-radioactive *in situ* hybridization exhibited cytoplasmatic expression within every cell type, including all osteocytes (Fig. 4A), osteoblasts (Fig. 4A), chondrocytes, and bone marrow cells (data not shown). Control hybridizations of control tibiae with the sense rRNA probe showed no signals (Fig. 4B).

Endogenous IGF-I mRNA expression in tissue

The control brain showed IGF-I mRNA expression in Purkinje cells of the cerebellum and in neurons of the medulla oblongata (data not shown). In the internal control, i.e. the growth plate, IGF-I mRNA was located in chondrocytes of the proliferative and the hypertrophic zone (Fig. 4C). In control tibiae, IGF-I mRNA was expressed in osteoblasts, which were situated against the surface of trabecular bone (Fig. 4C) and endocortical bone (Fig. 4E). IGF-I mRNA expression was also observed in osteocytes, which were lying within the first lamella at the endosteal side of the shaft (Fig. 4E) and some trabecular osteocytes (Fig. 4C). The endocortical osteocytes, which were located within the deeper lamellae (Fig. 4E), and the periosteal osteocytes (data not shown) did not express IGF-I mRNA. IGF-I mRNA was expressed in the intracortical endothelial cells of blood vessels (Fig. 4F) and in the periosteum of control tibiae (data not shown). Some cells of the bone marrow i.e. megakaryocytes, macrophages, and myeloid cells also expressed IGF-I mRNA (data not shown).

Control hybridization of tibiae sections with the corresponding sense RNA probe did not show signals (Fig. 4D).

Effect of mechanical loading on IGF-I mRNA expression in osteocytes of the tibia shaft

Mechanical loading induced IGF-I mRNA expression in osteocytes within multiple layers at the endosteal side of the shaft of the tibia in contrast to the contra-lateral control tibia, where IGF-I mRNA expression was only seen within the superficial layer at the endosteal side of the shaft (Fig. 4E and F). Quantitative analysis of the osteocytes within the endosteal side of the shaft showed a twofold increase of IGF-I mRNA expression 6 h after mechanical loading (Figs 4F and 5). The proportion of IGF-I mRNA-positive osteocytes was 29·3 ± 12·9% (mean ± S.D.) for loaded tibiae (n = 5), 16·7 ± 4·4% (mean ± S.D.) for sham-loaded tibiae (n = 5), and 14·7 ± 4·2% (mean ± S.D.) for contra-lateral control tibiae (n = 10; Fig. 5). Mechanical loading significantly increased the number of osteocytes, which express IGF-I mRNA (P < 0·01, load versus contra-lateral control and P < 0·05, load versus sham (Fig. 5)).

Effects of mechanical loading on IGF-I mRNA expression in osteoblasts, chondrocytes, and bone marrow cells

No differences in IGF-I mRNA expression between loaded, sham-loaded, and contra-lateral control tibiae were observed in the osteoblasts, chondrocytes, and bone marrow cells by semi-quantitative screening. No differences in morphology of the cells were observed between groups.

Discussion

This *in vivo* study showed that a single mechanical loading session was sufficient to increase lamellar bone formation at the endosteal side of loaded tibiae shaft and to increase the IGF-I mRNA synthesis in the endocortical osteocytes of loaded tibiae 6 h after a single period of mechanical stimulation with the four-point bending system. The twofold increase of IGF-I mRNA expression was restricted to osteocytes at the endosteal side of the shaft and the inner lamellae. Loaded tibiae exhibited a higher number of IGF-I mRNA-positive osteocytes within multiple endosteal lamellae than non-loaded tibiae, whereas sham-loaded tibiae showed a similar pattern of IGF-I mRNA expression in sub-endocortical osteocytes as non-loaded contra-lateral control tibiae. We conclude that the increase of IGF-I mRNA expression in the osteocytes is a consequence of bending, because squeezing of the tibiae during sham-loading did not influence the IGF-I mRNA expression pattern. We suggest that the IGF-I mRNA in the endocortical osteocytes plays a role in lamellar bone formation.

These results confirm previous findings. Lean *et al.* (1995) showed an increase of IGF-I mRNA in cortical and trabecular osteocytes of the eighth caudal vertebra 6 h after

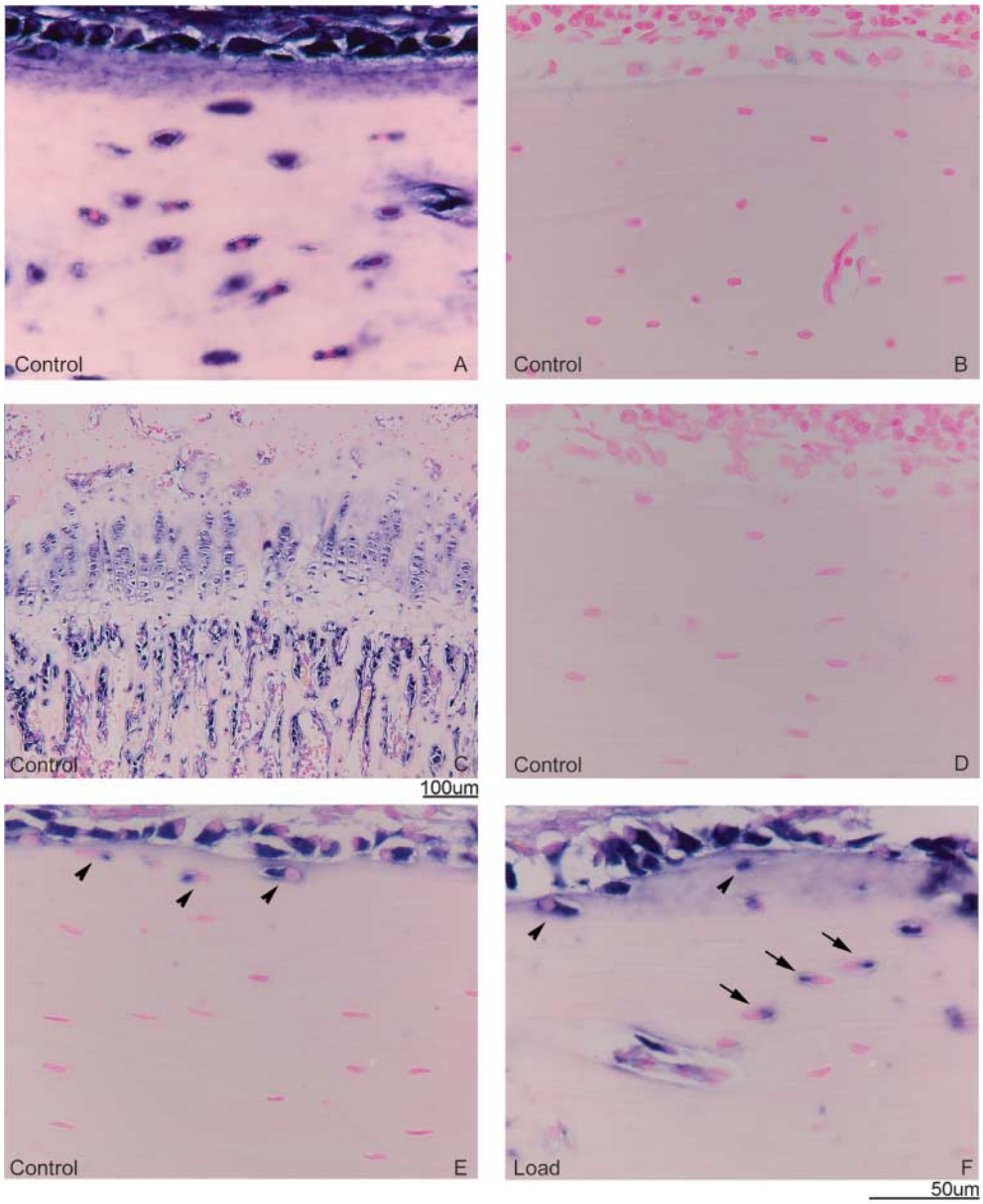

Figure 4 Expression of rRNA in (A) longitudinal control; tibiae sections and (C and E) IGF-I mRNA expression in longitudinal control; tibiae sections and (F) longitudinal loaded tibiae sections of 12-week-old female Wistar rats. The RNA signal is shown as a blue precipitate. The sections were counterstained with nuclear fast red and were visualized under brightfield illumination. The scale bars represent 50 μm (A and B, D, E and F) and 100 μm (C) respectively. (A) 28S rRNA expression within all endocortical osteocytes and osteoblasts; (B) hybridization with representative 28S ribosomal sense probe showed no signal in the endocortical osteocytes and the osteoblasts; (C) IGF-I mRNA expression in the growth plate and primary spongiosa, the trabecular osteocytes, and the trabecular osteoblasts; (D) hybridization with a representative IGF-I sense probe showed no signal in the endocortical osteocytes and the osteoblasts; (E) IGF-I mRNA expression within the superficial endocortical osteocytes (indicated by arrow heads) and the osteoblasts of a contra-lateral control tibia; and (F) IGF-I mRNA expression in the endocortical osteocytes within multiple layers (indicated by arrow heads and by arrows) and the osteoblasts of a loaded tibia.

mechanical stimulation with the invasive vertebra compression model demonstrated by *in situ* hybridization and Raab-Cullen *et al.* (1994) showed an increase of IGF-I mRNA in the periosteum 4 h after loading with the four-point bending model demonstrated by northern blot (Raab-Cullen *et al.* 1994). Besides, IGF-I protein concentration in bone is also increased after stimulation by mechanical stress using additional weight-bearing

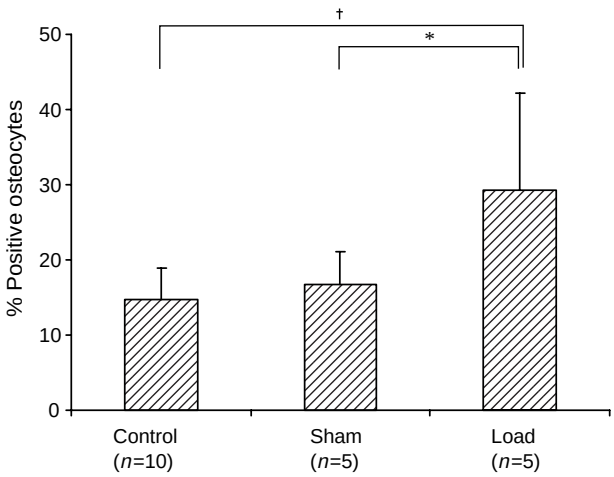

Figure 5 Influence of mechanical loading on IGF-I mRNA expression in the osteocytes at the endosteal side of the shaft of rat tibiae. Values are expressed as mean percentage positive osteocytes ± S.D. (†$P<0{\cdot}01$, load versus contra-lateral control; *$P<0{\cdot}05$, load versus sham).

(rat-with-backpack) as was previously reported (Bravenboer *et al.* 2001). The current study is the first study which showed a twofold increase of IGF-I mRNA in osteocytes in a non-invasive mechanical loading model (four-point bending model) at cellular level *in vivo* (non-radioactive *in situ* hybridization).

In addition, the twofold increase of IGF-I mRNA was specifically detected in the osteocytes at the endosteal side of the shaft within the deeper lamellae. It has generally been accepted that osteocytes are responsible for the predominant sensing of mechanical strains in bone (Klein-Nulend *et al.* 1995). It can be concluded that the osteocytes at the endosteal side of the shaft and the inner lamellae are mechanosensitive, since these osteocytes synthesize IGF-I mRNA 6 h after mechanical loading. This is also shown by Gross *et al.* (2002) who reported that mice overexpressing IGF-I in osteoblasts had an increased periosteal bone formation, suggesting that IGF-I increased sensitivity of the osteocytes and the osteoblasts.

Furthermore, we suggest that the mechanosensitive osteocytes, which synthesize IGF-I mRNA 6 h after mechanical loading, are important for the increased lamellar bone formation after mechanical loading. Forwood *et al.* (1996) reported that a single short period of loading, using the four-point bending model, resulted in an increased lamellar bone formation rate at the endosteal surface of rat tibia. 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 and increased lamellar bone formation rate at the endosteal surface (Forwood *et al.* 1998). This has been confirmed in the validation study at our laboratory which showed that a single mechanical loading session was sufficient to induce lamellar bone formation at the endosteal side of the tibiae and the woven bone at the periosteal bone side. The lamellar bone formation is located at the endosteal side of the shaft, which is similar to the location of newly synthesized IGF-I mRNA after mechanical loading. This study also showed that IGF-I mRNA expression was not observed in the osteocytes at the periosteal side of the shaft, but was restricted to the sub-endocortical osteocytes and the osteocytes in multiple lamellae. The IGF-I mRNA may be less involved by the woven bone formation, which is the result of irritation of the periosteum (Forwood *et al.* 1998). This suggests that IGF-I mRNA in the mechanosensitive osteocytes is specifically important for lamellar bone formation.

No differences in serum IGF-I concentrations were observed between loaded, sham-loaded, and control groups. This implies that the acute effect of mechanical loading is restricted to the bone region, which is deformed, because the systemic serum IGF-I concentrations are unaffected. This supports the hypothesis that the osteogenic response to mechanical loading occurs locally (Bravenboer *et al.* 2001). However, the level of IGF-I mRNA, which has to be produced at a single skeletal site to observe the effect on IGF-I protein in the serum, is unknown. A twofold up-regulation of IGF-I mRNA in one tibia might be too low.

The other bone cells in the tibiae and the bone marrow cells showed no difference in IGF-I mRNA expression before and 6 h after mechanical loading. Our results showed that osteoblasts and osteocytes, within the first lamella at the endosteal side of the shaft and the trabecular bone, synthesize IGF-I mRNA. These bone cells play a role during bone remodeling. Although the osteoblasts are responsible for new bone formation after loading, their IGF-I mRNA expression was not increased after mechanical stimulation; this is probably due to the fact that the endogenous IGF-I mRNA expression level in osteoblasts was very high. Several investigators have also demonstrated endogenous IGF-I mRNA expression within the osteocytes of rodents (Inaoka *et al.* 1995, Mason *et al.* 1996, Zhao *et al.* 2000). In contrast, a number of other studies did not observe endogenous IGF-I mRNA expression within the osteocytes of rodents and humans (Yeh *et al.* 1993, Lean *et al.* 1995, Middleton *et al.* 1995). These contradictory results could be explained by the fact that different bones, including tibiae, distal femurs, ulnae, and vertebrae, were examined. The daily loading of these bones varies considerably, which results in differences in gene expression. A second explanation could be the use of various molecular techniques and the preparation of the bone tissue, either undecalcified bone or decalcified bone. Finally, there is a difference in age between the studied species.

For this study, we used the four-point bending model of Forwood, because this four-point bending 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 (Turner *et al.* 1991, Forwood *et al.* 1998). The osteogenic response will occur locally in bone. Therefore, we have used the *in situ* hybridization technique in order to detect the local osteogenic response at the cellular level. The non-radioactive *in situ* hybridization method is a powerful and

sensitive technique method to localize gene expression within decalcified rat tibiae. In this study, a ribosomal 28S RNA probe was used to verify the RNA integrity of the decalcified tibiae. All bone cells, including osteocytes and osteoblasts, and chondrocytes and bone marrow cells showed ribosomal 28S RNA expression within the cytoplasm. Therefore, we conclude that the RNA integrity was maintained during the entire decalcification and embedding procedure. It is demonstrated that the IGF-I cRNA probe is specific, because the brain showed IGF-I mRNA expression in the Purkinje cells of the cerebellum and in neurons of the medulla oblongata as described earlier (Bondy *et al.* 1992, D'Ercole *et al.* 1996, Reijnders *et al.* 2004) and IGF-I mRNA was expressed in the chondrocytes of the proliferative and the hypertrophic zone of the growth plate as shown earlier by Reinecke and Nilsson (Nilsson *et al.* 1990, Reinecke *et al.* 2000).

Nevertheless, this study has some limitations. The applied load of 60 N is supra-physiological and the insulin-like growth factor-binding proteins (IGFBPs) have not been studied. IGF-I is one component of the IGF system. IGFBPs can influence the biological activity of IGF-I (Firth & Baxter 2002) by regulating the bioavailability of IGFs (Collett-Solberg & Cohen 1996). Therefore, it is necessary to study the effects of mechanical loading on local IGFBP expression level as well.

In conclusion, this study shows that IGF-I mRNA is twofold up-regulated within the endocortical osteocytes of the shaft and multiple layers extending into the cortical bone 6 h after mechanical loading *in vivo*. We conclude that these osteocytes are mechanosensitive as shown by newly synthesized IGF-I mRNA after a single short period of loading. This supports the hypothesis that these osteocytes translate mechanical stimuli into bone formation through IGF-I. The process occurs rather early in a series of cellular events, which take place after mechanical loading.

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