Influence of intermittent administration of parathyroid hormone on muscle tissue and bone healing in orchietomized rats or controls

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Abstract

Influence of human parathyroid hormone (hPTH 1–34) on muscle and bone healing was studied in either orchietomized (Orx at 8 months of age) or sham-operated male rats. Eleven-month-old Sprague–Dawley rats underwent bilateral transverse metaphyseal osteotomy of tibia and were divided into four groups ($n=12$): 1) sham-vehicle, 2) sham group-PTH everyday, 3) Orx-vehicle, 4) Orx-PTH everyday, and 5) Orx-PTH every other day. PTH dosage was 40 µg/kg body weight. After 5 weeks, fiber cross-sectional area, capillary density, and enzyme activity (lactate dehydrogenase, citrate synthase, and complex I) were measured in soleus (MS), gastrocnemius (MG), and longissimus (ML) muscles; tibiae were analyzed by computed tomographical, histological, and gene expression analyses. The effect of PTH in all rats was increased serum osteocalcin, cortical and callus

densities and callus area. In sham rats capillary density was increased in limb muscles (MS: 1.3–1.7, MG: 1.2–1.4 capillaries/fiber), and rate of osseous bridging of osteotomy was enhanced (67–100%). In Orx rats serum creatine kinase was decreased (6670–2847 U/l), and bone genes (*Igf-1*, osteoprotegerin, and receptor activator of nuclear factor κB ligand) were up-regulated. Cross-sectional area, enzyme activity, food intake, weight of body, visceral organs, adipose tissue, MG, and MS were not affected by PTH. PTH had a favorable effect on muscle capillary density and improved bone healing being more effective in sham rats and having no adverse systemic effect. The effect was less if PTH was applied every other day. The findings may show up trends for therapeutic treatment of male patients.

Journal of Endocrinology (2011) **209**, 9–19

Introduction

Intermittently administered human parathyroid hormone (hPTH (1–34)) is the only clinically approved bone anabolic therapy for osteoporotic patients of both genders (Gagnon *et al.* 2008). PTH regulates serum calcium by acting mainly on bone and kidney and stimulating calcium re-absorption and bone resorption in the situation of low serum calcium levels (Partridge *et al.* 2006). Intermittent exposure to PTH as once daily injections increased bone formation over resorption. Recent studies have demonstrated that intermittent administration of PTH enhanced fracture healing in experimental animals (Barnes *et al.* 2008, Komrakova *et al.* 2010). Since the osteoporotic are most commonly seen as postmenopausal and senile, the majority of research on osteoporosis has focused on women, though it is also becoming an important problem in men. Hypogonadism in young men and age-related decline in gonadal hormone levels in older men are associated with the development of senile osteoporosis

(Gagnon *et al.* 2008). Gonadal androgens exert a wide variety of physiological and morphological effects in organisms (Meisel & Sachs 1994). Androgen deficiency causes the loss of muscle weight and induces atrophy in muscles (Jiang & Klueber 1989). Testosterone replacement increases skeletal muscle mass and lowers body fat levels in hypogonadal men (Bhasin *et al.* 1997).

Applying PTH to improve bone quality or fracture healing in osteoporotic organism, it is also important to investigate the effects of PTH treatment on muscle tissue that is functionally linked to the bones. A single injection of PTH caused a rapid decrease in serum calcium followed by a rapid recovery to baseline values (Frolik *et al.* 2003). As calcium has a central role in muscle function, it is conceivable that alternations of serum calcium levels affect muscle tissue. Furthermore, excess of PTH itself appears to have detrimental effects on skeletal muscle metabolism, since it has been shown that hyperparathyroidism is associated with muscle dysfunction (Shin & Stern 1967, Garber 1983, Baczynski *et al.* 1985). A reason

might be that PTH impairs production, transfer, and utilization of energy in rat skeletal muscle (Baczynski *et al.* 1985). High serum levels of PTH were associated with loss of muscle strength and muscle mass in humans (Visser *et al.* 2003). In a recent study in our working group, it was shown that PTH administered intermittently had no effect on mitochondrial respiratory activity and muscle structure of gastrocnemius muscle in both healthy and ovariectomized (Ovx) female rats of 6 months of age (Komrakova *et al.* 2010). However, it is known that aging affects muscle tissue differently in females and males (Phillips *et al.* 1993). Furthermore, gender- and muscle-specific differences were observed in the regulation of fiber types by thyroid hormones (Larsson & Yu 1997). After PTH administration, these differences may also be found. We are not aware of the influence of PTH applied to improve fracture healing on skeletal muscles in male organism.

The present study was undertaken to investigate the effect of PTH administration on structure and metabolism of soleus (MS), gastrocnemius (MG), and longissimus (ML) muscles of 12-month-old male rats either orchiectomized (Orx) or sham operated undergoing tibia-healing period. The current study gives an account of bone-healing process.

Materials and Methods

The animal study protocol was approved by the local regional government and conformed to German animal protection laws (permission from 12/05/03, Az: 509.42502/01-53.03 District Government of Braunschweig). All chemicals were obtained from Sigma-Aldrich Chemie GmbH unless otherwise indicated.

Sixty 8-month-old male Sprague-Dawley rats were obtained from Janvier (Le Genest-Saint-Isle, France). Under i.p. ketamine (Medistar, Holzwickede, Germany) and xylazine (Riemsers, Greifswald, Germany) anesthesia (60 mg and 8 mg/kg of body weight (BW) respectively), 24 rats were sham operated (sham), and 36 rats were bilaterally Orx. Rats were housed three to four in standard cages under 12 h light:12 h darkness regimes at a constant temperature of $22 \pm 1^\circ\text{C}$. Rats received standard pelleted diet (Ssniff special diet GmbH, Soest, Germany) throughout the experiment. BW of rats and food intake were recorded weekly. All animals had free access to food and water throughout the experiment.

After 12 weeks (Fig. 1), all rats underwent a bilateral metaphyseal osteotomy of tibia (Stuerner *et al.* 2010). Briefly, under ketamine/xylazine anesthesia, rats received subcutaneously decantan (Merck) and rimadyl (Pfizer, Karlsruhe, Germany) (5 mg and 4 mg/kg BW respectively). Thereafter, a transverse osteotomy was created using an ultrasound saw (Piezosurgery, Mectron Medical Technology, Carasco, Italy). Tibia fragments were fixed with the aid of a T-titan plate and four screws (Stryker Trauma, Selzach, Switzerland).

Figure 1 Schematic flowchart of the experiment. Eight-month-old male rats were either orchiectomized (Orx) or sham operated (week 0). After 3 months, all rats underwent bilateral osteotomy of tibia (Ost, week 12) and were divided into groups ($n=12$) that were either treated with s.c. PTH (40 $\mu\text{g/kg}$ BW per day) everyday (PTH/d) or every other day (PTH/2d) or treated with vehicle for up to 5 weeks (week 17). Dotted line, sham orchiectomy; dashed line, Orx; continuous line, PTH/d; dot-and-dash line, PTH/2d; n , number of rats survived to the end of experiment.

After osteotomy, rats were divided into five groups (12 rats each). In group 1, sham-operated rats were given vehicle everyday (0.9% NaCl; sham). In group 2, sham-operated rats were treated with PTH everyday (sham PTH/d). In group 3, Orx rats were given vehicle (Orx). In group 4, Orx rats were treated with PTH everyday (Orx PTH/d). In group 5, Orx rats were given PTH every other day (Orx PTH/2d). All injections were done subcutaneously from the first day after osteotomy. A single dosage of PTH was 40 $\mu\text{g/kg}$ BW. Newly formed callus was labeled by fluorescence dyes, which were injected subcutaneously (Rahn 1976). Xylenol orange (90 mg/kg BW) was injected on day 13, calcein green (10 mg/kg BW) was injected on day 18, and alizarin complexone (30 mg/kg BW) was injected on days 22 and 24 after osteotomy respectively. Tetracycline (25 mg/kg BW) was injected 1 h prior to decapitation, i.e. 5 weeks after osteotomy (Fig. 1).

Blood samples (0.5–1.0 ml) were collected at the time of orchiectomy and osteotomy by penetrating the retro-orbital sinus with a glass capillary tube and used for the analyses of serum testosterone level.

Animals were decapitated under CO_2 anesthesia 5 weeks after osteotomy (Fig. 1). Blood samples were collected for analyses of serum testosterone, osteocalcin (Oc), alkaline phosphatase (Alp), and creatine kinase (CK) levels. Heart, liver, kidney, spleen, and visceral adipose tissue were extracted and weighed. The presence of testes has been additionally checked.

Samples of MG, MS and ML were randomly collected from either left or right side immediately after decapitation of rats. A block of ML (1 cm^3) was excised at the level of the last rib. The entire MG and MS were carefully excised, weighted, pinned at both ends, and cut in the middle across the muscle. Thereafter, muscle samples were removed from the center of the ML, MG, and MS, frozen directly in liquid nitrogen, and stored at -80°C until either histological or enzyme analyses. The three different muscles were chosen because of their different muscle fiber-type composition. Enzyme analyses

were performed only with samples from the ML and MS, as these muscles have different localizations and clearly differ in muscle fiber composition (Schwartz-Giblin *et al.* 1983, Armstrong & Phelps 1984).

Tibiae either left or right were dissected and freed of soft tissues. Plates and screws were extracted. One tibia chosen randomly was stored at -20°C for flat-panel volume-computed tomographic (fpVCT) and histomorphological analyses. Metaphyseal clips of other tibia containing newly formed callus were stored at -80°C for gene expression analyses.

Serum analyses

Serum analyses were conducted at the Department of Clinical Chemistry, University of Goettingen. Serum testosterone level was measured using liquid chromatography tandem mass spectrometry with the aid of a Quattro Premier XE Micromass-System (Waters Corp., Milford, MA, USA) (Streit *et al.* 2007). Serum CK was detected by the 'reverse reaction' and activation by acetylcysteine. OC was determined by electrochemiluminescence immunoassay analyses and ALP by colorimetric assay. Analyses were done using automated chemistry analyzer (Roche/Hitachi Modular) and commercially available kits (Roche) according to the manufacturer's instructions (Roche Diagnostics GmbH). *In vitro* assays are assigned for determination of CK, ALP, and OC in human serum. Inter-assay coefficient of variation (CV) was 0.7, 1.4, and 1.5%, and intra-assay CV was 0.6, 0.6, and 0.7% for ALP, CK, and OC assays respectively (Roche). Sensitivity was 0.67 U/l, 3 U/l, and 0.50 ng/ml for ALP, CK, and OC respectively (Roche).

Analyses of muscle fibers and capillary density

Serial cross sections of $12\text{ }\mu\text{m}$ were cut from the removed samples of the three muscles using a cryostat (Leica, Frigocut 2800E, Nussloch, Germany) at -20°C and mounted on glass slides. For the analysis of muscle fibers, sections were fixed in 1% paraformaldehyde solution (pH 6.6) containing 1% CaCl_2 and 6% sucrose and then stained by incubation in reduced NADH-diaphorase solution (pH 7.4) followed by acidic incubation (pH 4.2) and incubation in adenosine-5'-triphosphate solution (pH 9.4) (Horák 1983). Muscle fibers were classified into fast-twitch glycolytic (G), fast-twitch oxidative, and slow-twitch oxidative according to Peter *et al.* (1972). The two oxidative (O) fiber types were not always clearly distinguished and, therefore, were analyzed together. The MS mainly consists of O fibers (Armstrong & Phelps 1984). Cross-sectional area of at least 60 O and G fibers determined within two fields of the cross section (30 fibers per field) was measured.

For the analysis of muscle capillaries, sections were fixed for 1 h in 100% ethanol/chloroform/glacial acid at a ratio of 16:3:1 at 4°C . Thereafter, PAS staining method (Andersen 1975) was applied to stain the blood vessels. Briefly, after

incubation in 0.3% α -amylase (from porcine pancreas) solution, sections were treated with 1% periodic acid and stained in Schiff's Reagent solution (Roth, Karlsruhe, Germany). Finally, sections were treated with 10% potassium sulfite solution. Capillaries and fibers found in three randomly selected fields (1 mm^2 each) within cross section were counted. The ratio of capillaries to muscle fiber was taken in the analyses.

The measurements were done with the aid of a digital camera (DVC 1310 C, DVC Company, Maidenhead, UK) and a microscope (Eclipse E 600, Nikon, Japan) at a magnification of $\times 10$ using a computerized image analysis program (Lucia G, Laboratory Imaging, Version 4.82, Praha, Czech Republic).

Enzyme analyses

Muscle samples were weighed and homogenized in ice-cold Chappell-Perry medium (0.1 M KCl, 0.05 M Tris, 0.01 M $\text{MgCl}_2 \times 6\text{H}_2\text{O}$, and 1 mM EGTA, pH 7.5) using a Potter-S-homogenisator (B.Braun Biotech International, Melsungen, Germany) and kept on ice between measurements. Aliquots were frozen for measurements of protein content. Enzyme activities were assayed using a spectrophotometer (Spectronic Genesys 2PC, Pittsford, NY, USA) and WinSpec software (Spectronic Instruments). The assays were performed in triplicates at 30°C over 5 min and a pH of 7.4 unless otherwise indicated. The analysis was done within 2 h after tissue had been initially homogenized. The enzyme activities of the lactate dehydrogenase (LDH, EC 1.1.1.27) are expressed in U/mg protein and the enzyme activities of the citrate synthase (CS, EC 2.3.3.1) and NADH-ubiquinone oxidoreductase (complex I, EC 1.6.5.3) are expressed in U/g protein.

In glycolysis, LDH catalyzes the reduction of pyruvate (1.2 mM) to lactate accompanied with the oxidation of NADH (150 μM) to NAD^+ . In the enzyme activity assay, the oxidation of NADH results in the time-dependent reduction of absorption at 340 nm, as NAD^+ shows no absorption at this wavelength.

CS activity was assayed according to Faloona & Srere (1969). In the citrate cycle, CS catalyzes the condensation of acetyl-coenzyme A (CoA) (0.1 mM) and oxalacetate (0.5 mM) to citrate accompanied with the liberation of CoA. In the enzyme activity assay, the liberated CoA molecules irreversibly bind to 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB, 0.1 mM) forming thionitrobenzoic acid. The time-dependent increase of the latter product was followed at 412 nm, as the thionitrobenzoic acid absorbs the light at this wavelength. The detergent Triton X-100 (0.1%) was added to the incubation solution for disintegration of all mitochondria and liberation of all CS proteins.

The complex I activity was assayed according to Hatefi & Stiggall (1978). In the electron transport chain, complex I catalyzes the oxidation of NADH (0.2 mM) to NAD^+ accompanied with the reduction of ubiquinone (CoQ)

(0.12 mM) to dihydroubiquinone (CoQH₂). The enzyme activity was analyzed at 340 nm. The assay was initially performed over 10 min, and subsequently, the complex I inhibitor rotenone (10 µM) was added to incubation solution before the assay was carried out for a further 10 min. Rotenone was added to control the complex I-independent processes of NADH reduction. Complex I activity was calculated by deducting the enzyme activity with rotenone from the total activity. To inhibit further steps of the electron transport chain, the complex III inhibitor antimycin A (1.0 µM) and cytochrome oxidase inhibitor potassium cyanide (1 mM) were added to the incubation solution.

Protein content was determined in frozen-thawed aliquot using multilabel reader (Perkin Elmer Precisely Victor X4, Turku, Finland) and software version 4.0 (Perkin Elmer Life and Analytical Science). BCA Protein Assay Kit based on bicinchoninic acid for the colorimetric detection and quantification of proteins was used according to the manufacturer's protocol (Pierce, Rockford, IL, USA).

Analyses of bone healing

FpVCT analysis has been done *in vitro* after extraction of plate and screws. Tibia was thawed and analyzed using an fpVC tomograph (GE Global Research, Niskayuna, NY, USA), a non-clinical CT prototype (Missbach-Guentner *et al.* 2007). Data analysis was done on an Advantage Workstation (version 4.2, General Electric Health Care, Milwaukee, WI, USA). The measurement area extended 2 mm proximally

and 2 mm distally from the osteotomy site. Callus and cortical (Cl. and Ct.) densities were initially assessed in Hounsfield units (HU) and then converted into bone mineral density (Cl.BMD and Ct.BMD, mg/cm³) with the aid of hydroxyapatite standards of several mineral densities.

Thereafter, the entire tibia was subjected to the sequential ascending concentrations of ethanol and embedded in methylmethacrylate (Merck). Sections of 150 µm thickness were cut longitudinally at right angle to the implant bed using Leica SP 1600 diamond saw microtome (Leica Instruments GmbH, Nussloch, Germany). Osseous bridging of the osteotomy gap was analyzed using at least 10 fluorochrome labeled sections (Fig. 2) with the aid of a Leica microscope (Leitz DM RXE; Leica Mikroskopie & Systeme GmbH, Wetzlar, Germany). Calcein green stained area was built within 13 and 18 days after osteotomy, alizarin complexone-stained tissue was formed from 18 to 24 days, and tetracycline-stained tissue was formed within 24 and 35 days after osteotomy (Komrakova *et al.* 2010).

Three representative central sections of the tibia were digitalized using a digital camera (Leica DC300F) and a zoom stereomicroscope (Leica MZ75) at a magnification of ×16 with the aid of QWin image analysis program (Leica, Bensheim, Germany). The measurement area was similar to that assessed in fpVCT analyses. It was divided into three regions of interest: 1) plate side, ventral aspect; 2) dorsal aspect; 3) the endosteal part of the tibia (Fig. 2a–e). Callus area (µm²) was measured according to the regions of interest.

Figure 2 Images of longitudinal sections of tibia metaphysis labeled with fluorochromes made 35 days after osteotomy in treatment groups: (a) sham; (b) sham PTH/d; (c) Orx; (d) Orx PTH/d; (e) Orx PTH/2d. Arrowheads, ventro-medial aspect; arrows, dorsal aspect; rectangular frame, region of interest.

Analyses of bone gene expression

Metaphyseal clips of the contralateral tibia containing newly formed callus were homogenized using micro-dismembrator S (Sartorius, Goettingen, Germany). Total cellular RNA was extracted using the RNeasy Mini Kit (Qiagen) and quantified with the aid of a photometer (Biometra, Goettingen, Germany). Purity of RNA was checked based on the absorbance ratio at 260 and 280 nm, which ranged from 1.9 to 2.2. The RNA samples (100 ng) were then reverse transcribed using Superscript RNase H⁻ reverse transcriptase (Promega). Expression of rat genes, *Alp1*, *Oc*, insulin-like growth factor-1 (*Igf-1*), receptor activator of nuclear factor κ B ligand (*Rankl*), osteoprotegerin (*Opg*), and collagen type 1 alpha 1 (*Col1 α 1*) were determined by quantitative real-time PCR (qRT-PCR) based on SYBR Green detection using iCycler (CFX96, Bio-Rad Laboratories). Ready-to-use primer pairs were obtained from Qiagen (QuantiTect Primer Assays). The qRT-PCR was carried out in 25 μ l volume containing 12.5 μ l QuantiTect SYBR Green PCR Kit (Qiagen), 1 μ l primer pairs, and 2 μ l cDNA. PCR profile was 1 cycle at 95 °C for 15 min, 40 cycles at 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s, followed by 80 cycles at 55–95 °C for 30 s where set point temperature increased after cycle 2 by 0.5 °C. Amplification efficiencies for gene of interest and reference gene were around 100%. Relative gene expression was calculated using $2^{-\Delta\Delta C_T}$ method (Livak & Schmittgen 2001) for each gene of interest, where $\Delta\Delta C_T = (C_T \text{ treatment group gene} - C_T \text{ reference gene } \beta\text{-2 microglobulin (Mg)}) - (C_T \text{ intact group gene} - C_T \text{ reference gene } \beta\text{-2 MG})$. In intact group ($n=10$), male rats were intact (non-Orx, non-osteotomized, and untreated), maintained under the same conditions as the experimental animals, were of the same age, and had a comparable BW. Samples of tibia were collected from the metaphyseal part similar to that collected in osteotomized tibia. Gene expression in intact group was taken as 1.

Statistical analyses

Statistical analyses were conducted using SAS program (Version 9.0, SAS Institute, Cary, NC, USA). ANOVA (F -test, $P<0.05$) was applied to reveal the impact of the treatment on the respective variables. Additionally, the effect of duration was taken in the model analyzing food intake and BW variables. The weight of the rats was taken as a covariate to examine its effect on the variables. The effect of BW on weight of heart, liver, kidney, spleen, fat, MG, and MS was highly significant ($P<0.001$, F -test) and, therefore, organ to BW ratio (mg/g) was calculated and compared statistically. Differences between individual means were estimated using Scheffé test ($P<0.05$). Data are shown as means and S.E.M. Non-parametric Kruskal–Wallis test and Dunn's multiple comparison test were used for analyses of gene expression (GraphPad Prism 4.0, GraphPad Software, San Diego, CA, USA).

Results

Serum analyses

Serum testosterone was at the similar level among the rats at the beginning of the study (Table 1). Thereafter, it declined in all groups. In Orx rats, it dropped drastically to undetectable levels in some animals. In sham rats, testosterone level decreased gradually, but was significantly higher than that measured in Orx rats.

OC level significantly increased after PTH administration in all rats (Table 1). The highest level was measured in 'Orx PTH/d' group. PTH applied with less frequency (Orx PTH/2d) showed less effect on OC level; however, it was still at a higher level than that in untreated rats. The activity of ALP did not change significantly after PTH treatments in all rats ($P>0.05$).

CK levels decreased significantly in 'Orx PTH/d' group compared with that measured in Orx, sham, and 'sham PTH/d' groups (Table 1). The differences in CK level between Orx and sham rats were not significant ($P>0.05$).

Table 1 Serum testosterone level in orchietomized (Orx) or sham rats treated either with PTH everyday (PTH/d), every other day (PTH/2d) or given vehicle measured at the day of orchietomy (Orx, 8 months of age), osteotomy (11 months of age), and killing (12 months of age); serum osteocalcin (OC), alkaline phosphatase (ALP), and creatine kinase (CK) levels were measured after killing. At least ten replications per treatment group were done

	Sham		Sham PTH/d		Orx		Orx PTH/d		Orx PTH/2d	
	Mean	S.E.M.	Mean	S.E.M.	Mean	S.E.M.	Mean	S.E.M.	Mean	S.E.M.
Testosterone (ng/ml)										
Orx	2.3 [*]	0.5	2.6 [*]	0.2	2.2 [*]	0.6	2.2 [*]	0.6	2.6 [*]	0.6
Osteotomy	1.8 ^{*,†,§}	0.3	1.7 ^{*,†,§}	0.3	0.02 ^{†,§}	0.004	0.03 ^{†,§}	0.01	0.02 ^{†,§}	0.001
Killing	1.2 ^{*,§}	0.2	1.1 ^{*,§}	0.1	0.02 ^{†,§}	0.001	0.03 ^{†,§}	0.003	0.03 ^{†,§}	0.002
OC (μ g/l)	15 [*]	1.2	47	2.8	18 [*]	1.5	56 [†]	2	31	1.3
ALP (U/l)	115	11	133	7	113	7	131	7	134	8
CK (U/l)	9542 [*]	1539	6881 ^{*,}	917	6670 ^{*,}	1357	2847 [†]	1237	5900 ^{†,}	737

^{*,†,||,§}Within row, between the treatment groups, means with different superscripts differ significantly ($P<0.05$, Scheffé test). ^{*,§}Within column, between testosterone levels at different time points, means with different superscripts differ significantly ($P<0.05$, Scheffé test).

Figure 3 Body weight (g) of orchiectomized (Orx) or sham operated (week 0) rats which were treated after osteotomy (week 12) with PTH everyday (PTH/d) or every other day (PTH/2d) or given vehicle for up to 5 weeks (week 17). Data are presented as means and S.E.M. (abc) Between weeks, means with different letters differ significantly, showed combined for all groups; (*) between all sham versus all Orx, means are significantly different; (#) between ‘sham’ alone versus all Orx, between ‘sham PTH/d’ versus Orx alone and ‘Orx PTH/2d’, means are different at the respective week ($P<0.05$, Scheffé test).

Food intake, BW, and organ weight

Between the treatment groups, food intake did not differ significantly during first 9 weeks after orchiectomy (data not shown). During further weeks, Orx rats consumed less food compared to the sham animals (week 10, 24 vs 29; week 17, 25 vs 30 g/rat per day on average respectively).

PTH treatments did not affect food consumption ($P>0.05$). In all rats, a significant rise was observed in food intake during 3 weeks after orchiectomy followed by a decrease at week 4 (week 1, 20 ± 2 ; week 3, 43 ± 1 ; week 4, 30 ± 1 g/rat per day on average respectively). Thereafter, it remained without conspicuous changes to the end of the experiment with the exception of week 13 (week following osteotomy) when daily intake dropped to 16 ± 2 g per day on average.

BW of Orx rats was significantly lower than that of sham rats from the first week after orchiectomy onward (Fig. 3). In group ‘Orx PTH/d’, BW was lower compared with that in ‘sham PTH/d’ group during experiment; however, the differences reached significant level from week 7. BW decreased ($P<0.05$) in Orx rats after orchiectomy during the first week, then rose to the initial level observed at Orx during the next 2 weeks, and remained unchanged up to week 12 (Fig. 3). The weight of sham rats continuously rose over 4 weeks and then remained without significant changes. After osteotomy (week 13), it dropped significantly in all groups, slightly recovering in sham groups and remaining at a lower level in Orx rats towards the end of the experiment. PTH treatments had no effect on BW of all rats.

Weight of heart, kidney, spleen, visceral adipose tissue, MG, and MS did not differ significantly between the groups in absolute and relative terms (Table 2). Liver was significantly heavier in sham-operated rats compared to that in Orx rats irrespective of PTH treatments.

Muscle fibers and capillary density

Muscle fiber size in ML and MG did not differ significantly among the groups (Table 3). In MS, cross-sectional area of

Table 2 Final wet weight of body, organs, adipose tissue, and muscles of orchiectomized (Orx) or sham rats either given vehicle or treated with PTH everyday (PTH/d) or every other day (PTH/2d) during 5 weeks of tibia healing. At least ten replications per treatment group were done

	Sham		Sham PTH/d		Orx		Orx PTH/d		Orx PTH/2d	
	Mean	S.E.M.	Mean	S.E.M.	Mean	S.E.M.	Mean	S.E.M.	Mean	S.E.M.
Weight (g)										
Heart	1.8	0.1	1.7	0.2	1.5	0.1	1.4	0.1	1.5	0.04
Liver	22.0*	1.0	20.6*	0.7	15.6 [†]	0.7	16.1 [†]	0.7	15.6 [†]	0.7
Kidney	4.1	0.2	4.0	0.2	3.2	0.1	3.1	0.1	3.2	0.4
Spleen	1.5	0.1	1.5	0.1	1.4	0.1	1.2	0.04	1.2	0.1
Adipose tissue	20.3	2.0	17.4	2.0	19.0	2.9	18.9	3.2	18.1	2.0
MG	3.7	0.1	3.5	0.1	3.2	0.1	3.3	0.1	3.3	0.1
MS	0.3	0.01	0.3	0.02	0.3	0.01	0.3	0.01	0.3	0.01
BW	710*	25	685*	19	603 [†]	19	613 [†]	25	587 [†]	20
Organ to BW ratio (mg/g)										
Heart	2.7	0.1	2.5	0.1	2.5	0.1	2.4	0.1	2.5	0.2
Liver	32.0*	1.2	30.0*	0.5	26.7 [†]	0.7	26.8 [†]	0.6	27.1 [†]	0.1
Kidney	6.0	0.2	5.9	0.2	5.5	0.2	5.2	0.2	5.7	0.2
Spleen	2.1	0.1	2.2	0.1	2.1	0.3	2.0	0.1	2.0	0.1
Adipose tissue	29.3	2.5	25.0	2.5	30.8	4.0	29.9	3.3	29.1	2.5
MG	5.4	0.3	5.1	0.2	5.1	0.5	5.3	0.2	5.5	0.2
MS	0.5	0.02	0.5	0.02	0.5	0.1	0.5	0.02	0.5	0.03

*[†]Between the treatment groups, means with different superscripts differ significantly ($P<0.05$, Scheffé test).

Table 3 Cross-sectional area (CSA, μm^2) of glycolytic (G) and oxidative (O) fibers, and number of capillaries per muscle fiber in gastrocnemius (MG), longissimus (ML) and soleus (MS) muscles of orchietomized (Orx) or sham rats either given vehicle or treated with PTH everyday (PTH/d) or every other day (PTH/2d) during 5 weeks of tibia healing. At least seven replications per treatment group were done

	Sham		Sham PTH/d		Orx		Orx PTH/d		Orx PTH/2d	
	Mean	S.E.M.	Mean	S.E.M.	Mean	S.E.M.	Mean	S.E.M.	Mean	S.E.M.
Histological analyses										
ML										
CSA, G (μm^2)	7417	612	6204	509	5942	603	6183	454	6102	398
CSA, O (μm^2)	3557	366	2750	208	2811	287	2714	324	2594	192
Capillaries/fiber	1.3	0.04	1.2	0.1	1.2	0.1	1.3	0.1	1.2	0.03
MS										
CSA, O (μm^2)	5167 ^{*,†}	200	5788 [†]	412	4826 [*]	140	4759 [*]	268	4339 [*]	193
Capillaries/fiber	1.3 [*]	0.04	1.7 [†]	0.1	1.6 [†]	0.1	1.7 [†]	0.1	1.5 ^{*,†}	0.1
MG										
CSA, G (μm^2)	6228	507	6154	605	5320	565	5223	239	5951	439
CSA, O (μm^2)	3237	411	3673	421	2747	200	2358	158	3583	351
Capillaries/fiber	1.2 [*]	0.1	1.4 [†]	0.1	1.5 [†]	0.1	1.4 [†]	0.1	1.3 ^{*,†}	0.1

^{*,†}Between the treatment groups, means with different superscripts differ significantly ($P < 0.05$, Scheffé test).

oxidative fibers in 'sham PTH/d' group was significantly larger compared to all Orx rats. Treatment with PTH had no significant effect on the size of muscle fiber in all rats ($P > 0.05$).

Capillary density was significantly enhanced after PTH treatment in MG and MS of sham rats, reaching the level measured in Orx rats (Table 3). In Orx and 'Orx PTH/d' groups, capillary density was higher compared with that in sham group. In 'Orx PTH/2d' group, capillary density did not differ from that in other treatment groups. In ML, there was no difference in capillary density among the groups (Table 3).

Enzyme analyses

Activity of LDH was similar among the groups: 3.8 U/mg protein in ML and 1.8 U/mg protein in MS on average for all animals (Table 4). CS and complex I activities did not differ significantly among the groups in both muscles studied (Table 4).

Bone analyses

Bone healing occurred at the beginning of the reparative stage: callus bridged osteotomized bone and the osteotomy line could be clearly observed (Fig. 2a–e). PTH administration resulted in a formation of larger and denser callus (Fig. 2b, d, and e) compared to that formed in untreated sham and Orx rats (Fig. 2a and c). The osseous bridging of the osteotomy gap occurred on day 32 on average in 67% of rats in the 'sham' group, whereas, in the remaining 33% of rats, the osteotomy was not bridged. In 'sham PTH/d' group, bridging was observed on day 28 on average in all rats (100%). In 'Orx' and 'Orx PTH/d' groups, the gap was bridged on day 27 on average in 82% and 90% of rats respectively. In 'Orx PTH/2d' group, bridging was on day 29 in 73% of rats.

Callus area was larger in rats treated with PTH on a daily basis compared with that in untreated groups (Table 5). Application of PTH every other day did not change callus area significantly. Sham rats had larger osseous callus area compared with that of Orx rats.

Table 4 Activity of lactate dehydrogenase (LDH), citrate synthase (CS), and complex I in longissimus (ML) and soleus (MS) muscles of orchietomized (Orx) or sham rats either given vehicle or treated with PTH everyday (PTH/d) or every other day (PTH/2d) during 5 weeks of tibia healing. Five replications per treatment group were done. Significant differences were not detected

	Sham		Sham PTH/d		Orx		Orx PTH/d		Orx PTH/2d	
	Mean	S.E.M.	Mean	S.E.M.	Mean	S.E.M.	Mean	S.E.M.	Mean	S.E.M.
Enzymes										
ML										
LDH (U/mg protein)	3.6	0.8	4.0	0.6	4.0	0.3	3.7	0.3	3.9	0.5
CS (U/g protein)	36.6	9.8	51.6	14.3	39.1	6.3	56.0	6.1	45.0	5.9
Complex I (U/g protein)	11.8	2.0	10.7	1.5	8.2	1.2	10.3	1.4	9.7	1.3
MS										
LDH (U/mg protein)	2.0	0.1	2.1	0.3	1.7	0.1	1.8	0.2	1.6	0.1
CS (U/g protein)	75.7	15.8	87.7	20.0	82.2	13.4	96.6	12.7	90.2	4.0
Complex I (U/g protein)	21.4	4.6	28.9	3.0	27.8	1.2	20.9	3.9	21.3	3.8

Table 5 Flat-panel volume-computed tomographic (fpVCT) and fluorescence analyses of tibia at the osteotomy site at the ventral and dorsal aspects and endosteally in sham or orchiectomized (Orx) rats either given vehicle or treated with PTH everyday (PTH/d) or every other day (PTH/2d) during 5 weeks of tibia healing. At least nine replications per treatment group were done

	Sham		Sham PTH/d		Orx		Orx PTH/d		Orx PTH/2d	
	Mean	S.E.M.	Mean	S.E.M.	Mean	S.E.M.	Mean	S.E.M.	Mean	S.E.M.
Parameters										
<i>fpVCT</i>										
Ct.BMD (mg/cm ³)	740*	19	797 [†]	19	729*	14	774 [†]	17	795 [†]	16
Cl.BMD (mg/cm ³)	328*	13	438 [†]	19	327*	8	448 [†]	35	410 [†]	16
Fluorescence labeled callus area (µm ²)										
Ventral	976* [†]	172	1815 [‡]	258	666*	115	1492 ^{†,‡}	249	1205* ^{†,‡}	349
Dorsal	4140* [†]	450	5021 [†]	400	3146*	542	6738 [†]	496	3546*	394
Endosteal	6219*	401	6870*	624	4502 [†]	316	6528*	528	4310 [†]	416
Total	11 334*	581	13 706 [‡]	680	8313 [†]	611	14 758 [†]	656	9060 [†]	644

*[†][‡]Between the treatment groups, means with different superscripts differ significantly ($P < 0.05$, Scheffé test). BMD, bone mineral density; Ct, cortical; Cl, callus.

Callus and cortical densities measured by fpVCT were significantly higher in all rats treated with PTH compared with those in untreated rats (Table 5). The differences between sham and Orx rats in Ct. and Cl.BMD were not significant.

In general, there were little differences in response of bone markers to PTH treatment. However, *Opg*, *Igf-1*, and *Rankl* gene expression changed significantly in PTH-treated rats (Fig. 4). *Igf-1* and *Rankl* genes in ‘Orx PTH/2d’ group were significantly up-regulated ($P < 0.05$) comparing with those in control sham and Orx groups (Fig. 4d and e). *Opg* gene in ‘Orx PTH/d’ group had significantly higher expression compared to that in the sham group (Fig. 4e). Orchiectomy had less effect on the expression of bone markers.

Discussion

PTH is a recently prescribed treatment for severe osteoporosis in women and men. However, the influence of PTH has been analyzed predominantly in females and is insufficiently investigated in males (Gagnon *et al.* 2008). The patients are often diagnosed as osteoporotic when the osteoporotic fractures occurred. Since the osteoporotic fractures typically occurred in the metaphysis, the metaphyseal tibia osteotomy was chosen, and bone healing was analyzed in osteopenic and healthy male rats. The study was designed to mimic the clinical situation, where osteoporotic patients would receive PTH treatment to improve bone healing. PTH treatment augments bone mass in osteoporotic patients (Gagnon *et al.* 2008), enhances fracture healing in experimental animals (Barnes *et al.* 2008, Komrakova *et al.* 2010), and has no adverse effect on structure of gastrocnemius muscle in 6-month-old female rats (Komrakova *et al.* 2010). As age-, gender-, and muscle-specific differences could be found in the response to PTH treatment, the effect of PTH on metabolic and structural properties of the two limb muscles gastrocnemius and soleus and the back muscle longissimus was analyzed. Rats display deleterious changes in bone 4 weeks after

orchiectomy (Turner *et al.* 1989) and represent a valuable experimental model for osteoporotic studies (Wink & Felts 1980). The clear decrease in serum testosterone verified the success of orchiectomy in the present study. A slight decrease in testosterone level also in sham-operated rats was due to aging as also indicated by Bethea & Walker (1979).

Considering the structure of the investigated skeletal muscles, we found out that PTH and orchiectomy generally did not affect fiber size in male rats, although a decreasing influence of the orchiectomy could be found. PTH is known to increase intracellular calcium concentration in muscle tissue, which may disrupt muscle structure and exert an adverse effect on muscle metabolism (Garber 1983, Baczyński *et al.* 1985, Visser *et al.* 2003). Excess of calcium ions in sarcoplasm of organisms possessing malignant hyperthermia leads to a malignant hyperthermia crisis. If the process cannot be terminated, muscle tissue gets severely damaged (Berchtold *et al.* 2000). Elevated level of PTH in patients presenting hyperparathyroidism was associated with muscle weakness and reduced muscle mass (Visser *et al.* 2003). However, the different effect of continuous and intermittent exposure to PTH is recognized (Dobnig & Turner 1997, Frolik *et al.* 2003). Continuous infusions of PTH result in severe hypercalcemia in rat. After single injection, PTH reaches its maximum serum concentration within 15 min and, thereafter, is cleared from the circulation within 6 h (Dobnig & Turner 1997).

In the present study, BW and muscle fiber size decreased in Orx rats, whereas wet muscle weight did not change. Considering that wet muscle weight is affected by extracellular fluid accumulation and/or increased muscle fat content (Malenfant *et al.* 2001, McClung *et al.* 2006), it could be suggested that muscle tissue of Orx rats is replaced by fat tissue and water accompanied with fiber size reduction. However, further investigations are necessary.

The analysis of capillary density revealed that PTH enhanced ratio of capillaries to muscle fiber in gastrocnemius and soleus muscles in sham rats, whereas in Orx rats, PTH had

Figure 4 Box plot illustrating relative mRNA expression level of (a) *Oc*, (b) *Alp*, (c) *Col1α1*, (d) *Igf-1*, (e) *Opg*, and (f) *Rankl* genes. On the X-axis, treatment groups such as (1) sham, (2) 'sham PTH/d', (3) Orx, (4) 'Orx PTH/d', and (5) 'Orx PTH/2d' are calculated using $2^{-\Delta\Delta C_T}$ method relative to gene expression in untreated rats taken as 1. Each treatment was conducted with at least eight replications. Medians with different letters differ significantly ($P < 0.05$, Dunn test).

minor effect. The generally higher capillary density of Orx rats compared to sham rats seems to be related to the decrease of the muscle fiber sizes. Parathyroid tissue is capable of inducing revascularization after implanting it in muscle (Wells *et al.* 1977). *In vitro*, parathyroid tissue significantly augmented angiogenesis at lower microvessel densities, whereas, at higher densities, there were no significant differences (Garter *et al.* 1996). Garter *et al.* (1996) proposed that there is equilibrium of factors that control angiogenesis. When the equilibrium is shifted towards a more angiostatic state, for instance at lower capillary density in sham group in the present study, a PTH may be capable of shifting the equilibrium towards angiogenesis. Under more angiogenic conditions, such as higher capillary density in Orx rats, PTH influence may be lost. Furthermore, angiogenesis is a complex process influenced by multiple factors that rarely occurs in the absence of disease or injury (Folkman & Klagsbrun 1987). Wound fluid and certain prostaglandins, which levels were elevated in wounds and inflammatory exudates, have been

shown to be angiogenic (Folkman & Klagsbrun 1987). The latter might explain why the differences in capillary density were observed in the limb muscles, which were close to the surgery location at the tibia and not in the back muscle.

In the present study, a significant decrease of CK level was observed in serum of Orx rats treated with PTH everyday. The enhanced level of serum CK is indicative of muscle injury, myocardial infarction, muscular dystrophy, and malignant hyperthermia. CK regulates the intracellular concentration of ATP by catalyzing the generation of ATP from ADP and phosphocreatine or converts ATP into phosphocreatine. It was demonstrated that in cultured bone cells, PTH is able to increase CK activity (Sömjen *et al.* 1984). Reduced CK level may be found as a consequence of diminished efflux of the muscle enzyme into serum from reduced physical activity (in Orx rats) or may result from reduced muscle mass (fiber size in the present study) accompanying Orx (Rosalski 1998). Studying metabolic enzymes involved in glycolysis, citrate cycle, and oxidative phosphorylation, no adverse effect of PTH was revealed on functional cell properties and, therefore, on muscle tissue performance as well.

PTH administration increased callus area and density, as well as cortical density in both Orx and sham rats when it was applied on a daily basis, whereas, every other day application improved solely density and had no effect on callus area. In contrast, in Ovx 6-month-old female rats, PTH improved osteotomy healing irrespectively of administration regimes (Komrakova *et al.* 2010). Age- and gender-specific differences may be responsible for different PTH impact in bone healing. PTH has been reported to enhance osteogenesis in experimental fracture healing (Nakajima *et al.* 2002). PTH increased percentage of rats possessing osteotomy bridging and accelerated osteotomy bridging more in sham rats than in Orx. A similar response was observed in female rats (Komrakova *et al.* 2010), supporting the statement that stimulation of bone healing by PTH is more effective in healthy organisms with unaltered levels of gonadal hormones than in testosterone/estrogen-deficient rats. Although fracture healing is supposed to be impaired in osteoporotic organisms (Namkung-Matthai *et al.* 2001, Stuermer *et al.* 2010), bone bridging in Orx rats occurred earlier than in sham rats (days 27 vs 32). Decreased callus area in Orx rats may be a consequence of diminished bone formation rate and periosteal apposition rate reported in rats after orchiectomy (Turner *et al.* 1989). Elevated serum OC level in all animals treated with PTH indicated increased osteoblast activity that may promote further bone synthesis and bone healing. However, enhancement of OC level was less if the PTH was applied with less frequency (everyday versus every other day). The sensitivity of serum OC to PTH-induced changes in bone turnover has been shown previously (Komrakova *et al.* 2010).

Interestingly, PTH administration caused up-regulation of osteoblast and osteoclast genes reaching significant levels in Orx rats (*Opg*, *Igf-1*, and *Rankl* mRNA gene expression). Investigating bone genes in the callus 5 weeks after osteotomy

in 6-month-old female rats, neither PTH nor ovariectomy influenced mRNA gene expression (Komrakova *et al.* 2010). Based on the previous study (Meyer *et al.* 2003), it was suggested that changes in gene expression are measurable during early stages of healing and are not detectable 5 weeks after osteotomy. Since fracture healing takes longer in older animals (Meyer *et al.* 2003), it is possible that in 12-month-old rats, the healing was slowed and the differences in mRNA gene expression were still seen. Lack of androgens could additionally affect osteotomy healing and gene expression. In agreement with previous studies, mRNA expression level of *Oc* gene did not correspond to the serum level of *Oc* (Komrakova *et al.* 2010).

Increased muscular capillary density and in bone, earlier callus bridging, enhanced gene response to PTH in Orx rats may be an adaptation to the changes stimulated by orchietomy (e.g. increased bone turnover) (Delmas *et al.* 2000, Gennary & Bilezikian 2007).

BW, food intake, weight of adipose tissue, visceral organs, and muscles of male rats were observed to be unaffected by PTH treatment. These data support the different effects of chronic and intermittent exposure to PTH. It is known that PTH serum level is positively correlated with BW and fat mass in humans (Bolland *et al.* 2006). The lower weight measured in Orx rats throughout the experiment is in agreement with earlier observations that withdrawal of androgens by orchietomy decreases weight gain and food intake in male rats (Gentry & Wade 1976). In our study, food intake and BW did not change in parallel. Food intake was reduced in Orx rats after week 13 onward. As previously reported, BW did not directly reflect food intake (Gentry & Wade 1976), and some metabolic changes (Wade & Gray 1979) and bone loss (Wink & Felts 1980) may reduce BW in Orx rats. Orchietomy decreases physical activity and muscle mass, and increases carcass fat content in male rats (Wade & Gray 1979). Moreau *et al.* (2001) found no modification of fat mass 16 weeks after orchietomy. In the present study, the weight of visceral adipose tissue, visceral organs and muscle did not differ significantly among the groups. Weight of the liver in sham rats was higher than that of Orx rats. Castration has been reported to reduce levels of hepatic lipids as well as liver weight (Takeuchi *et al.* 1986).

In summary, intermittent PTH administration had no adverse effect on enzymes involved in muscle metabolism. PTH either lowered levels of serum CK in Orx rats or did not change levels in sham rats, indicating unimpaired muscle structure and function. PTH also stimulated vascularization in limb muscles of sham rats which had lower capillary density. PTH treatment increased callus area and cortical and callus densities in all rats, improved tibia healing in sham rats, which had lower rate of osteotomy bridging and up-regulated some bone genes in the callus of Orx rats. Administration of PTH with less frequency (PTH/2d) proved to be of limited success and is not recommended. PTH had no adverse effect on muscle structure, food intake, BW, nor weight of visceral adipose tissue and organs. Concluding, PTH applied daily

appeared to have favorable effects on muscle tissue and bone healing in both hypogonadal and healthy male rats. However, there is concern that the changes related to the lack of androgens might blunt the effect of PTH to some extent. The findings may have important implications for therapeutic treatment of male patients.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

The present study was supported by the AO International Foundation (S-100).

Acknowledgements

The authors thank F Kauer, R Castro-Machguth, A Witt, and R Wigger for their help.

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Received in final form 7 January 2011

Accepted 13 January 2011

Made available online as an Accepted Preprint
13 January 2011