

Changes in osteopontin and in biomarkers of bone turnover during human endotoxemia

Gabriele Grimm^a, Greisa Vila^b, Christian Bieglmayer^a, Michaela Riedl^b, Anton Luger^{b,*}, Martin Clodi^b

^a Department of Medical and Chemical Laboratory Diagnostics, Medical University of Vienna, Austria

^b Division of Endocrinology and Metabolism, Department of Internal Medicine III, Medical University of Vienna, Waehringer Guertel 18-20, A-1090, Vienna, Austria

ARTICLE INFO

Article history:

Received 18 December 2009

Revised 23 February 2010

Accepted 20 April 2010

Available online 24 April 2010

Keywords:

Bacterial endotoxin

Bone markers

Osteopontin

Human studies

First-phase response

ABSTRACT

Systemic infection and inflammation in men are associated with bone loss. Rodent studies have elucidated the pathways mediating the effects of bacterial lipopolysaccharide (LPS), activated immune cells and hormones on bone. Here we investigate the changes in biochemical parameters of bone turnover following human endotoxemia, an experimental model of self-limiting systemic infection and inflammation.

Ten healthy men received in a randomised, placebo-controlled, cross-over trial once placebo and once 2 ng/kg *Escherichia coli* endotoxin (LPS). During the following 6 h we monitored parathyroid hormone (PTH) and osteopontin (OPN), a multifunctional protein related to bone pathophysiology, as well as biochemical markers of bone turnover: C-terminal telopeptide of type I collagen (CTX), N-terminal propeptide of type I collagen (P1NP) and osteocalcin (OC). In LPS sessions there was a transient fall in PTH at 3 h ($p = 0.009$) and a nearly two-fold increase in OPN levels after 6 h (LPS: 155 ± 19 pg/ml; placebo: 85 ± 13 pg/ml, $p < 0.001$). LPS gradually reduced CTX levels (LPS: 0.44 ± 0.4 pg/ml; placebo: 0.59 ± 0.06 pg/ml, $p = 0.003$), P1NP showed a peak at 4 h (LPS: 89.9 ± 14.7 pg/ml; placebo: 75 ± 9.7 pg/ml, $p = 0.028$) and circulating OC did not change.

The early human response to systemic endotoxemia boosts osteopontin levels and modifies bone biomarkers, indicating a decrease in the lytic activity of osteoclasts, accompanied by an increase in the activity of immature osteoblasts. These changes might present the acute phase response of immune and bone cells to bacterial stimuli in men.

© 2010 Elsevier Inc. All rights reserved.

Introduction

Bone remodelling is the result of the proliferative activity of osteoblasts leading to bone formation and the catalytic activity of osteoclasts leading to bone loss [1]. The equilibrium between these two processes is controlled by a concert of immunoendocrine factors, but also by the nutritional status and mechanical factors [2]. Increased bone turnover and bone loss accompany chronic systemic infection and inflammation [3]. Metabolic bone disease may complicate severe sepsis and septic shock, both demanding challenges in intensive care units [4]. The underlying pathophysiological mechanisms include direct, cytokine-mediated and hormone-mediated effects of bacterial components on bone cells [4].

Bone remodelling processes are under the influence of several hormones, the action of which might be modulated by osteopontin (OPN), a multifunctional protein with cytokine properties produced mainly by immune cells, osteoblasts, osteoclasts, endothelial and

epithelial cells [5,6]. The dynamics of bone formation and bone resorption processes can be assessed by immunoassays that measure the amount of bone-originating proteins [7]. C-terminal telopeptide of type I collagen (CTX) is a sensitive marker of bone loss [8]. N-terminal propeptide of type I collagen (P1NP) and osteocalcin (OC) reflect the function of osteoblasts [8,9]. Based on these biochemical markers, both balance and rate of bone turnover can be estimated simultaneously by the recently proposed “bone marker plot” [10].

The intravenous administration of LPS in men induces a self-limiting febrile response and constitutes a reliable model for studying the pathophysiological mechanisms of human infection and inflammation [11]. The increase in body temperature is accompanied by a surge in circulating cytokines and neuroendocrine hormones that mimics that of sepsis [12]. Critically ill patients display increased biomarkers of bone turnover. This suggests not only an increased activity of osteoclasts, but also an increased activity of immature osteoblasts [13]. The signalling pathways activated by bacterial endotoxin (lipopolysaccharide, LPS) in bone cells have been extensively investigated in vitro as well as in rodent studies in vivo. To our knowledge, no relevant experimental studies have been performed in humans. Thus we investigated in a randomised, placebo-controlled cross-over study the impact of human endotoxemia on circulating

* Corresponding author. Fax: +43 140400 7790.

E-mail address: anton.luger@meduniwien.ac.at (A. Luger).

PTH and osteopontin levels as well as on biomarkers of bone resorption and bone formation.

Methods

Study subjects

The study was performed in compliance with the Declaration of Helsinki and approved by the Ethics Committee of the Medical University of Vienna. Ten men aged between 21 and 39 years old (mean 25.5 years) were recruited after prior written informed consent. All subjects presented neither a medical history nor clinical signs of acute or chronic disease and were taking no medication. Glucose, lipid values, C-reactive protein, as well as liver, kidney and thyroid function were normal as assessed via routine tests in a certified laboratory.

Design

The placebo-controlled, cross-over trial included two sessions, in which the participants received in a randomised design either placebo or lipopolysaccharide (LPS). Subjects came to the clinical research unit at about 8:00 after 12 h of fasting. Two indwelling catheters were placed in the antecubital veins of the right and left forearm, one used for infusions and the other for blood sampling. Placebo (0.9% NaCl) or 2 ng/kg body weight LPS (National Reference *Escherichia coli* endotoxin, U.S.P. Convention Inc., Rockville, MD) were administered in a bolus just after blood sampling at time point 0h, serving for baseline. The observation period lasted 6h after the LPS and placebo bolus. Temperature and vital signs were monitored continuously and blood samples were obtained hourly. Data on the LPS-induced changes in ghrelin, cortisol, ACTH and circulating cytokines obtained from the same study were previously published [14].

Assays

Blood samples for PTH, OPN, OC, CTX and P1NP analyses were taken in tubes containing EDTA, which were immediately cooled on ice and centrifuged within 30 min. Plasma samples were stored deep frozen at -80°C . Samples belonging to one individual (obtained in two different study sessions) were analysed within one analytical run and in duplicates. "Intact" PTH (1–84 PTH and cross-reactivity with N-terminal PTH fragments), OC, CTX, and P1NP were measured by electrochemiluminescence immunoassays on an Elecsys 2010 system (Roche Diagnostics, Germany) as previously described [15]. The intra- and interassay coefficients of variation were: 6–8% for PTH, 4–6 % for OC, 4 % for CTX and 2–5% for P1NP. OPN concentrations were determined using a commercial enzyme-linked immunosorbent assay (#DOST00, R&D Systems, Minneapolis, MN). The intraassay and interassay coefficients of variation were 2.9 and 5.4%, respectively.

Statistical analysis

Results are expressed as mean \pm standard error of mean (SEM). The Kolmogorov–Smirnov test was used to test the normality of data. The significance of differences between placebo and LPS treatments was determined using repeated measurements analysis of variance (ANOVA), with the interaction of time and treatment (time*treatment) being the term of interest. When ANOVA was positive, *post hoc* statistics was performed by means of paired *t*-tests for comparing the LPS-induced changes at specific time points. The SPSS release 12.0.1 software (SPSS, Inc., Chicago, IL) was used for statistical analysis and $p < 0.05$ was considered significant.

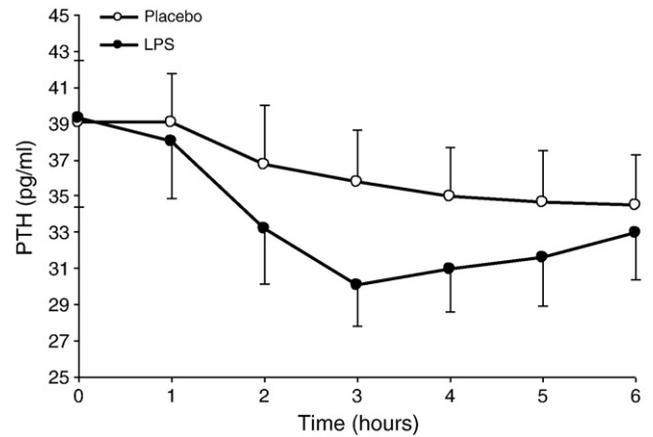


Fig. 1. PTH changes in response to LPS and placebo. The administration of LPS (2 ng/kg at time point 0 h, filled symbols) transiently decreases plasma PTH when compared to placebo (open symbols). Data are presented as mean \pm SEM.

Mathematical conversion of bone biomarker data for assessing bone turnover

The calculations were slightly modified from the recently described "bone marker plot" [10]. Briefly, for each marker the average marker concentrations at different time points were divided by the average concentration at the baseline time point 0h. These multiples of the starting level are independent from the respective scaling of marker concentrations and served as surrogates for formation (*F*) and resorption (*R*) forces which act on the bone (at baseline the forces are $F = 1$ and $R = 1$ by normalisation). Obviously, overall bone turnover is a resultant from formation and resorption forces. In analogy to the "parallelogram of forces" the resultant is a vector, which is mathematically formulated by its slope ($= F/R$) and length ($= \sqrt{F^2 + R^2}$), as obtained from the "Pythagoras theorem". The slope ($=$ ratio) describes the balance of formation and resorption processes. Conceivably, the vector's length is related to the rate of turnover. For a graphic presentation the logarithms of these transformed data are plotted, after shifting $\log(\text{length})$ by -0.15 to centre the data¹ (for further details see reference 10).

Results

LPS administration induced a transient decrease in plasma PTH ($p = 0.001$, ANOVA) reaching the lowest level of -16% at 3 h (30.1 ± 2.2 in LPS sessions versus 35.8 ± 2.8 in placebo sessions, $p = 0.009$, *post hoc* statistics) (Fig. 1). Circulating OPN concentrations increased from 65.6 ± 8.7 to 155.1 ± 19 pg/ml in the LPS sessions as compared to a changes from 67.3 ± 14.9 to 85.3 ± 13 pg/ml in the placebo sessions ($p < 0.001$, ANOVA) (Fig. 2). *Post hoc t*-tests were significant at time points 2 h ($p = 0.019$), 3 h ($p = 0.007$), 4 h ($p = 0.028$), 5 h ($p < 0.001$) and 6 h ($p < 0.001$). CTX concentrations decreased during the LPS session from 0.80 ± 0.05 ng/ml to 0.44 ± 0.04 ng/ml, as compared to changes from 0.69 ± 0.09 to 0.59 ± 0.06 in the placebo session ($p = 0.003$, ANOVA) (Fig. 3A). Significant changes were observed at time points 3 h ($p = 0.042$), 4 h ($p = 0.002$) and 6 h ($p = 0.016$). Plasma OC levels decreased by circadian variation ($p = 0.003$, ANOVA, effect of time), and these changes were not influenced by LPS treatment (Fig. 3B). P1NP significantly increased following LPS administration ($p = 0.028$, ANOVA), reaching the peak value of 89.9 ± 14.7 pg/ml at time point 4 h as compared to 75 ± 9.7 pg/ml in the placebo session ($p = 0.046$) (Fig. 3C). According to the biochemical bone markers, LPS caused a shift toward bone formation together with a decline in the rate

¹ With $F = 1$ and $R = 1$ at baseline, $\log(F/R) = \log[1] = 0$ and $\log \sqrt{(1+1)} = \log \sqrt{2} = \log(1.414) = 0.15$.

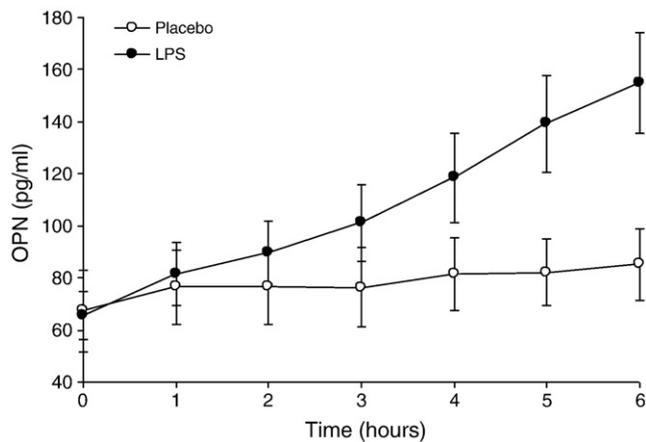


Fig. 2. Osteopontin changes in response to LPS and placebo. The administration of LPS (2 ng/kg at time point 0h, filled symbols) induces a continuous increase in plasma osteopontin when compared to placebo (open symbols). Data are presented as mean \pm SEM.

of bone turnover within 6h, contrasting to only minute changes during the placebo sessions (Figs. 4A and B). However, the formation markers behaved differently, because the reduction in bone turnover rate was more pronounced with OC, while the shift in balance toward bone formation was more pronounced with P1NP.

Discussion

The mechanisms underlying inflammatory bone disease have been extensively investigated in rodent studies. LPS exerts direct effects on bone cells, and modifies the levels of several hormones such as corticosteroids, known to influence bone function. In parallel, LPS activates immune cells and increases circulating concentrations of several cytokines, which modulate bone function [16–18]. The sum of chronic LPS effects on bone is an impaired equilibrium between bone formation and bone resorption leading to bone loss [3]. In humans, the relationship between inflammation and bone loss has been mainly investigated in patients with chronic inflammatory diseases, who present reduced bone mineral density and osteoporosis. Increased bone resorption is also found during prolonged hospitalisation [13]. Nonetheless, the increase in markers of bone turnover in intensive care unit patients does not correlate with circulating IL-6, IL-1, or TNF- α , suggesting the involvement of other mechanisms in acute bone disease [13].

Human endotoxemia is achieved by the intravenous infusion of LPS parts of *E. coli* and induces a self-limiting febrile, hormone and cytokine response. This experimental condition is extensively used for studies on innate immunity mechanisms in humans. During human endotoxemia we observed a transient fall in circulating PTH and a continuous increase in plasma osteopontin levels, suggesting early effects on bone turnover. Indeed, there were accompanying changes in biochemical markers of bone turnover characterised by a decrease in CTX levels, a transient increase in P1NP and no alteration in OC.

These profiles suggest a temporarily increased activity of immature osteoblasts, but an unchanged activity of mature osteoblasts, implying that the maturation of osteoblasts might be impaired following LPS administration in humans. The transient decrease in PTH, a hormone that stimulates bone resorption, is in line with the decrease of the bone resorption marker CTX, shifting the balance of bone remodelling toward bone formation at a reduced level of turnover rate.

Recent studies have elucidated a biphasic pattern in the neuroendocrine responses during critical illness [19]. The circulating levels of anterior pituitary hormones increase as part of the acute response, but decrease at a later stage [19]. Similarly, the profile of LPS-induced changes in hormones and cytokines varies during the response to LPS, with a transient increase in first response parameters (such as TNF- α) within 1–2h, followed by a later increase in secondary activation-

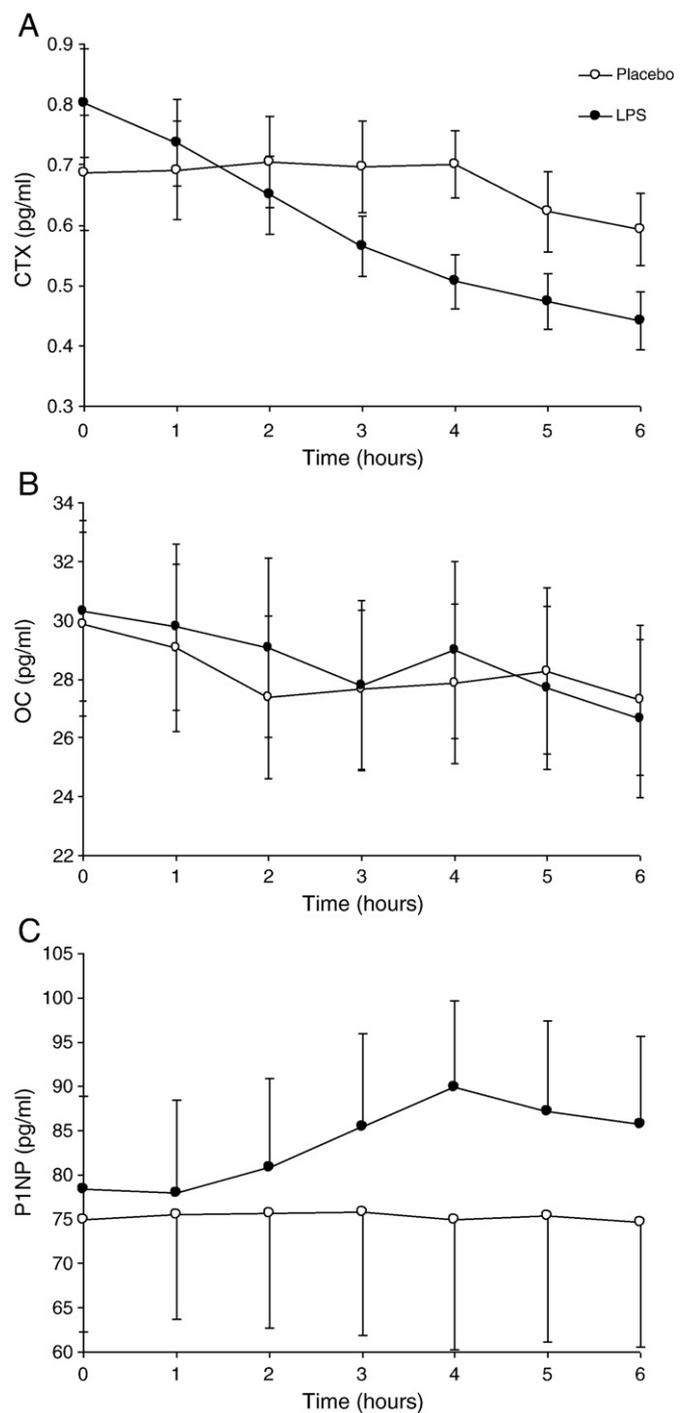


Fig. 3. Time course of biochemical markers of bone turnover following bolus infusions of LPS or placebo. Changes in CTX (A), OC (B) and P1NP (C) concentrations in response to LPS (2 ng/kg dosed at time point 0h, filled symbols) compared to placebo (open symbols). Data are presented as mean \pm SEM.

induced parameters (e.g. IL-1 receptor antagonist) [14]. Here we show that LPS acutely and transiently shifts the bone remodelling balance towards bone formation. The shift towards bone formation might be, like the increase in HPA axis activity, part of the evolutionary survival mechanisms, which aim to re-establish homeostasis and to eradicate the pathogen. The later decrease in HPA axis activity and bone loss found in chronic disease might reflect the exhaustion of primary response mechanisms.

The mechanisms underlying these LPS rapid effects, which are clearly opposite to the chronic effects on bone, may be multiple and

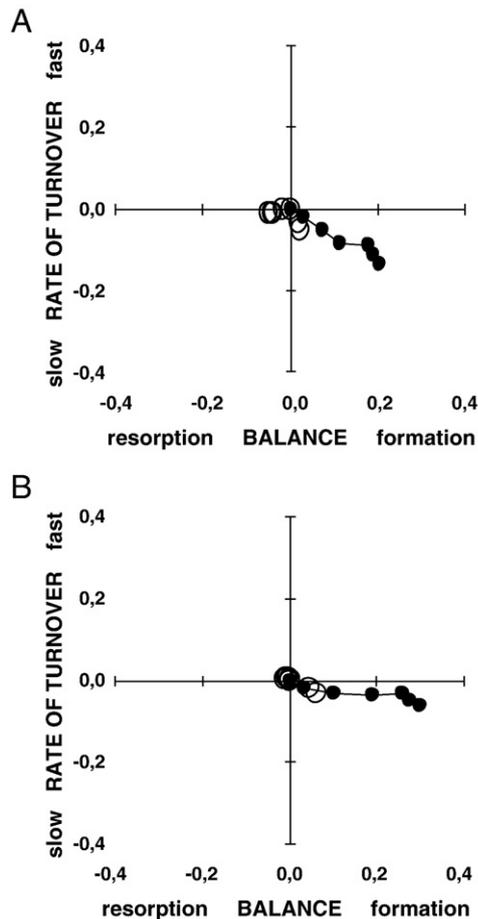


Fig. 4. Time course of the effects of LPS or placebo on overall bone turnover, shown by bone marker plots for marker combinations CTX & OC (A), CTX & P1NP (B). The average bone marker concentrations during 6 h of monitoring were normalised to the respective baseline levels (= centre of the plots). Open symbols: placebo, filled symbols: LPS.

interrelated. LPS has direct and dual effects on bone cells: inhibits or induces osteoclast differentiation depending on the experimental design in vitro (culturing conditions, paracrine factors, etc.) [20]. LPS may also affect the function of bone cells indirectly, as it modulates the function of immune cells and circulating levels of hormones and cytokines which are known to affect bone function [14,16–18]. The cross-talk of their rapidly activated signaling pathways at the molecular level might also count among the mechanisms mediating the shift towards bone formation.

Another novel finding of this study is the progressive increase in plasma OPN concentrations following LPS administration (Fig. 2). OPN is a signaling protein secreted by immune cells, epithelial cells, osteoblasts and osteoclasts. The transcription and secretion of OPN is increased in T-cells and macrophages in response to inflammatory stimuli [21,22], but also in cancer cells and during bone remodelling [5]. Clinically, elevated OPN levels are found in inflammatory diseases, sepsis and malignancy [23–25].

OPN binds to several receptors belonging to the integrin and CD44 families, thereby exerting multiple functions in different tissues [5]. Among others, OPN modulates immune responses, suppresses the action of PTH on bone and mediates calcitriol-induced bone resorption [5,6]. The increase in plasma OPN following LPS administration presented here is in line with the LPS-induced activation of immune cells. To date, there are no data on the direct influence of LPS on osteopontin transcription and secretion from osteoclasts and osteoblasts. The influence of increased plasma osteopontin levels on the processes of bone remodelling is also unclear. A possible link between circulating OPN and bone changes following infection/

inflammation remains to be investigated in future studies. Its multiple properties make OPN a candidate for interrelating the systemic response and the local skeleton changes following LPS administration.

In summary, LPS administration in men leads to an early and transitory increase in P1NP, decreases CTX and does not significantly change circulating OC concentrations. This constellation of changes suggests a transitory decrease in the lytic activity of osteoclasts, accompanied by an increase in the activity of immature osteoblasts. The shift of the balance of bone turnover towards bone formation together with a reduced rate of bone turnover might be part of the first human response to systemic infection and inflammation.

Acknowledgments

This study was supported by Research Grant 12323 of the Austrian National Bank (ÖNB).

References

- [1] Hadjidakis DJ, Androulakis II. Bone remodelling. *Ann N Y Acad Sci* 2006;1092:385–96.
- [2] Zaidi M. Skeletal remodeling in health and disease. *Nat Med* 2007;13:791–801.
- [3] Hardy R, Cooper MS. Bone loss in inflammatory disorders. *J Endocrinol* 2009;201:309–20.
- [4] Hollander JM, Mechanick JL. Bisphosphonates and the metabolic bone disease in the ICU. *Curr Opin Clin Nutr Metab Care* 2009;12:190–5.
- [5] Denhardt DT, Noda M, O'Regan W, Pavlin D, Berman JS. Osteopontin as a means to cope with environmental insults: regulation of inflammation, tissue remodeling, and cell survival. *J Clin Invest* 2001;107:1055–61.
- [6] Reinholt FP, Hultenby K, Oldberg A, Heinegard D. Osteopontin—a possible anchor of osteoclast to bone. *Proc Natl Acad Sci USA* 1990;87:4473–5.
- [7] Watts NB. Clinical utility of biochemical markers of bone remodeling. *Clin Chem* 1999;45:1359–68.
- [8] Seibel MJ. Biochemical markers of bone turnover. Part I: biochemistry and variability. *Clin Biochem Rev* 2005;26:97–122.
- [9] Fukumoto S, Martin TJ. Bone as an endocrine organ. *Trends Endocrinol Metab* 2009;20:230–6.
- [10] Bieglmayer C, Kudlacek S. The bone marker plot: an innovative method to assess bone turnover in women. *Eur J Clin Invest* 2009;39:230–8.
- [11] van der Crabben SN, Blümer SN, Blümer ME, Ackermans MT, Endert E, Tanck MW, Serlie MJ, van der Poll T, Sauerwein HP. Early endotoxemia increases peripheral and hepatic insulin sensitivity in healthy humans. *J Clin Endocrinol Metab* 2009;94:463–8.
- [12] Beishuizen A, Thijs LG. Endotoxin and the hypothalamo–pituitary–adrenal (HPA) axis. *J Endotoxin Res* 2003;9:3–24.
- [13] Van den Berghe G, Van Roosbroeck D, Vanhove P, Wouters PJ, De Pourcq L, Bouillon R. Bone turnover in prolonged critical illness: effect of vitamin D. *J Clin Endocrinol Metab* 2003;88:4623–32.
- [14] Vila G, Maier C, Riedl M, Nowotny P, Ludvik B, Luger A, Clodi M. Bacterial endotoxin induces biphasic changes in plasma ghrelin in healthy humans. *J Clin Endocrinol Metab* 2007;92:3930–4.
- [15] Chen Y, Bieglmayer C, Höcker P, Dettke M. Effect of acute citrate load on markers of bone metabolism in healthy volunteers. *Vox Sang Jun* 2009;97:324–9.
- [16] Clowes JA, Riggs BL, Khosla S. The role of the immune system in the pathophysiology of osteoporosis. *Immunol Rev* 2005;208:207–27.
- [17] Redlich K, Hayer S, Ricci R, David JP, Tohidast-Akrad M, Kollias G, Steiner G, Smolen JS, Wagner EF, Schett G. Osteoclasts are essential for TNF-alpha-mediated joint destruction. *J Clin Invest* 2002;110:1419–27.
- [18] Ozaki Y, Ukai T, Yamaguchi M, Yokoyama M, Haro ER, Yoshimoto M, Kaneko T, Yoshinaga M, Nakamura H, Shiraishi C, Hara Y. Locally administered T cells from mice immunized with lipopolysaccharide (LPS) accelerate LPS-induced bone resorption. *Bone* 2009;44:1169–76.
- [19] Vanhorebeek I, Van den Berghe G. The neuroendocrine response to critical illness is a dynamic process. *Crit Care Clin* 2006;22:1–15.
- [20] Zou W, Bar-Shavit Z. Dual modulation of osteoclast differentiation by lipopolysaccharide. *J Bone Miner Res* 2002;17:1211–8.
- [21] Ashkar S, Weber GF, Panoutsakopoulou V, Sanchirico ME, Jansson M, Zawaideh S, Rittling SR, Denhardt DT, Glimcher MJ, Cantor H. Eta-1 (osteopontin): an early component of type-1 (cell-mediated) immunity. *Science* 2000;287:860–4.
- [22] Guo H, Cai CQ, Schroeder RA, Kuo PC. Osteopontin is a negative feedback regulator of nitric oxide synthesis in murine macrophages. *J Immunol* 2001;166:1079–86.
- [23] Mishima R, Takeshima F, Sawai T, Ohba K, Ohnita K, Isomoto H, Omagari K, Mizuta Y, Ozono Y, Kohno S. High plasma osteopontin levels in patients with inflammatory bowel disease. *J Clin Gastroenterol* 2007;41:167–72.
- [24] Vaschetto R, Nicola S, Olivieri C, Boggio E, Piccolella F, Mesturini R, Damnotti F, Colombo D, Navalesi P, Della Corte F, Dianzani U, Chiochetti A. Serum levels of osteopontin are increased in SIRS and sepsis. *Intensive Care Med* 2008;34:2176–84.
- [25] Rittling SR, Chambers AF. Role of osteopontin in tumour progression. *Br J Cancer* 2004;90:1877–81.