

Bone structural and metabolic response of caloric restriction in Wistar rats and a GH-IGF-1 axis-suppressed transgenic rat model.

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The growth hormone–insulin-like growth factor-1 (GH–IGF-1) axis plays an important role in the effects of caloric restriction (CR) on lifespan extension and may elicit effects on bone metabolism in CR animals. We compared the effects of the GH–IGF-1 axis suppression and CR on bone metabolism. We used Wistar rats fed *ad libitum* (control group) or fed a 30% calorie-restricted diet in CR group and heterozygous transgenic (F1) rats whose GH-IGF-1 axis is moderately suppressed. There was no significant difference in serum IGF-1 concentration between control and CR rats; however, IGF-1 was significantly lower in F1 rats than in other groups. The bone volume fraction (BV/TV) was significantly lower in CR than in the control. The mean SMI value in CR rats was marginally significant difference from that in control rats, Although there was no difference in serum IGF-1 concentrations between CR and control rats, bone volume was lower, and higher SMI was observed in the former. The serum IGF-1 levels in F1 rats were lower than those of controls, but the bone volume and SMI in F1 were not different. Therefore, the effects of bone metabolism in CR rats may be different from those in the GH-IGF-1 suppression rats.

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Introduction

Previous studies have indicated that caloric restriction (CR) can extend the lifespan of several experimental animal models, including flies, mice and monkeys [1,2]. It has been suggested that this process is evolutionarily conserved in multiple organisms. At present, a 30% CR compared with *ad libitum* diets has been established as the most effective method for extending the lifespan of rodents. Studies using mutations that suppress the expression of *daf-2*, *age-1* and *daf-16*, the so-called 'long-lived' genes, have reported extensions in worm lifespans that are similar to those induced by CR [3,4]. The *daf-2*, *age-1* and *daf-16* encode genes corresponding to insulin / IGF-1 receptor, PI3K and forkhead type transcrip-

tion factor (Foxo) in mammals and those are present in the same signal pathway. It has also been shown that the growth hormone–insulin-like growth factor-1 (GH–IGF-1) axis is suppressed by CR, indicating that the GH–IGF-1 axis plays an important role in the effect of CR on lifespan extension.

Although several studies have reported an association of CR with the GH–IGF-1 axis and bone metabolism, this subject remains controversial. Previous studies have indicated that bone volume can be reduced by CR along with body weight reduction [5, 6]. Likewise, the GH–IGF-1 axis also plays an important role in bone metabolism. In humans, a significant association between a decline in serum IGF-1 concentration and bone volume has been reported [7]. The effects of CR on bone metabolism may be similar to those of

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GH-IGF-1 axis suppression. However, there are currently no reports comparing the change of animal bone metabolism caused by CR with that caused by GH-IGF-1 axis suppression. Shimokawa et al. reported a dwarf phenotype in a transgenic male Wistar rat model whose GH-IGF-1 axis was suppressed by overexpression of the antisense GH transgene (tg). The phenotype developed in heterozygous (tg/-) rats fed *ad libitum* (AL) and appeared similar to that of control non-transgenic (-/-) rats subjected to 30% CR [8]. In the present study, we used 10 month-old Wistar rats, fed *ad libitum* (control) or subjected to 30% CR (CR), and heterozygous (tg/-) rats (F1 rats) and examined their bone microstructure using micro-computed tomography (micro-CT) and measured serum concentrations of IGF-1 and bone-specific alkaline phosphatase (BAP). From the analysis of these results, we compared and discussed the effects of the GH-IGF-1 axis and CR on bone metabolism.

Materials and Methods

Animals

The present study was performed in accordance with the provisions of the Ethics Review Committee for Animal Experimentation at Nagasaki University (Japan). Rats and rat care used in the present study are described in detail elsewhere [8–10]. The transgenic rats (Jcl: Wistar-TgN (ARGH-GEN) 1Nts) were kindly provided by the Nippon Institute for Biological Science (Oume City, Tokyo, Japan). In the transgenic rats, the GH-IGF-1 axis is suppressed by the overexpression of the antisense GH gene. In brief, we used male Wistar rats purchased from Japan Clea Inc. (Tokyo, Japan) and male heterozygous transgenic (F1) rats (n=6).

After weaning at 4 weeks of age, rats were transferred to the barrier facility wherein the temperature (22°C–25°C) and light (12-hour light/dark cycle) were automatically controlled. Here all rats were fed *ad libitum* a Charles River LFP diet (Oriental Yeast Co. Ltd., Tsukuba, Japan), which is based on the Charles River CRF-1 formula; however, the protein fraction was reduced to 18%. The composition of the diet was as follows (per 100 g): 18.2 g protein, 4.8 g fat, 6.6 g mineral mixture, 5.0 g fibre, 57.9 g nitrogen-free water-soluble substances and 7.5 g water and the caloric value of the diet was 348 kcal/100 g.

At 6 weeks of age, Wistar rats were divided into two groups, control (n=6) and CR (n=6). Rats in the control and F1 groups continued to be fed *ad libitum*, while in the CR group a 30% CR regimen was initiated. The CR diet was administered by providing the rats with 140% of the mean

daily intake of control rats every other day until 24 weeks of age and then fixing the amount thereafter. All rats were euthanized by decapitation at 10 months of age.

Analysis of serum IGF-1 and BAP concentrations

After decapitation, trunk blood was collected and serum samples were stored at -30°C for later use. To measure serum IGF-1 and BAP concentrations, we used enzyme-linked immunosorbent assay (ELISA) kits specific for rat IGF-1 (Diagnostic Systems Laboratories Inc., Webster, TX, USA) and rat BAP (CUSABIO Biotech, China).

Micro-CT scanning

Micro-CT scanning was performed on the trabecular bone in the distal femur using a micro-CT-40 scanner (Scanco Medical AG, Bassersdorf, Switzerland). The distal femur was scanned using 240 slices with 16.4 µm increments at 55 Kvp and 145 µA. The scanning region was an area of trabecular bone about 200–4000 µm proximal from the distal epiphyseal line of the femur. On the original three-dimensional (3D) image, we directly determined morphometric indices, including the bone volume fraction (BV/TV), trabecular thickness (Tb.Th), trabecular number (Tb.N) and trabecular separation (Tb.Sp) from the binary volume of interest, using TRI/3D-BON bone structure measurement software (Ratoc System Engineering Co., Tokyo, Japan) [12]. As a nonmetric parameter, we also measured the structure model index (SMI). SMI reflects the relative proportion of plate- or rod-like structures in trabecular bone, with 0 indicating a plate-like structure and 3 indicating a rod-like one. A lower SMI value reflects a transition from a rod-like to a plate-like structure and increasing bone strength [12].

Statistical analysis

All data are expressed as the mean ± standard deviation of the mean. The averages of all data (body weight, serum IGF-1 and BAP concentration and micro CT scanning data) were compared using a Bonferroni test. Statistical analysis was performed using SPSS software version 16 (SPSS, Chicago, IL, USA). The level of statistical significance was established at $P < 0.017$ for all analyses.

Results

Body weight and serum IGF-1 and BAP concentrations

The mean body weight of CR rats (377.65 ± 22.13 g) was significantly smaller than that of controls (553.66 ± 10.51 g), and the body weight of F1 rats (329.01 ± 23.61 g) was markedly smaller than that of both control and CR rats (Fig. 1a). Differences were significant among all groups ($P < 0.001$). However, there was no significant difference between the mean serum IGF-1 concentrations of control (1419.0 ± 142.8

ng/ml) and CR rats (1289.4 ± 99.3 ng/ml), while the mean serum IGF-1 concentration of F1 rats (1007.2 ± 100.1 ng/ml) was significantly lower than those of the other two groups ($P < 0.01$; Fig. 1b). Furthermore, there was no significant difference in the mean serum BAP levels, a marker of bone formation, between any of the three groups (control, 49.7 ± 5.5 ng/ml; CR, 46.5 ± 4.5 ng/ml and F1, 38.9 ± 10.1 ng/ml; Fig. 2).

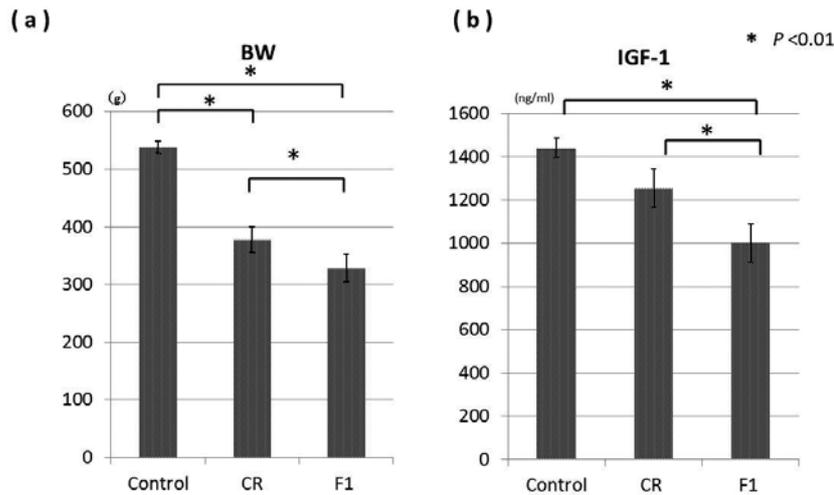


Figure 1: Body weight and serum IGF-1 concentration.

(a) The body weight of CR group was significantly smaller than that of Control group, and the body weight of F1 group was markedly smaller than that of both Control group and CR group ($P < 0.01$)
 (b) The serum IGF-1 concentration of F1 group (1007.2 ± 100.1 ng/ml) was significantly lower than Control group and CR group ($P < 0.01$).

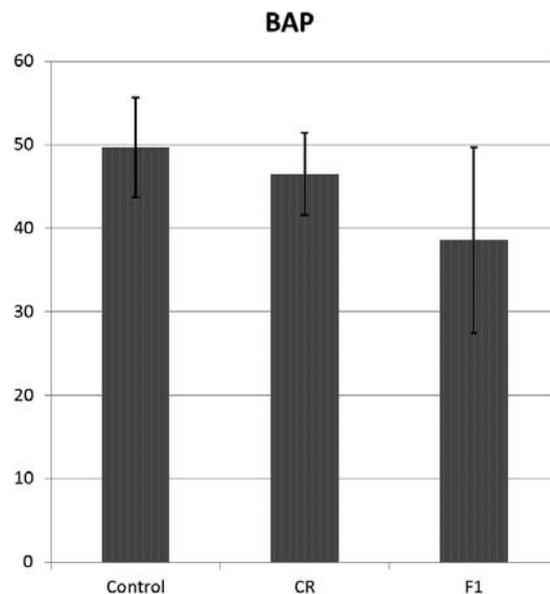


Figure 2: Serum BAP concentration.

there was no significant difference in the mean serum BAP level, a marker of bone formation, between any of the three groups.

Micro-CT scanning

The trabecular bone architecture at the condyle of the distal femur was measured with micro-CT. 3D-Micro-CT images showed that control rats have more and thicker trabecular bone mass than CR and F1 groups (Fig. 3). Mean BV/TV was significantly lower in CR rats ($15.5 \pm 2.1\%$) versus controls ($20.2 \pm 2.9\%$; $P = 0.013$); however, there was no significant difference between F1 ($16.9 \pm 2.0\%$) and either the

control or CR groups (Fig. 4a). There was also no significant difference in mean Tb.N, Tb.Th and Tb.SP among all three groups (Fig. 4b–d). The mean SMI value in CR rats (1.52 ± 0.25) was higher than that in control rats (1.15 ± 0.18), and there was marginally significant difference between two groups ($P = 0.021$). It was meaning a lower proportion of plate-like structures, leading to decreased bone strength in the CR group. However, there was no significant difference between control and F1 groups (1.24 ± 0.19 ; Fig. 4e).

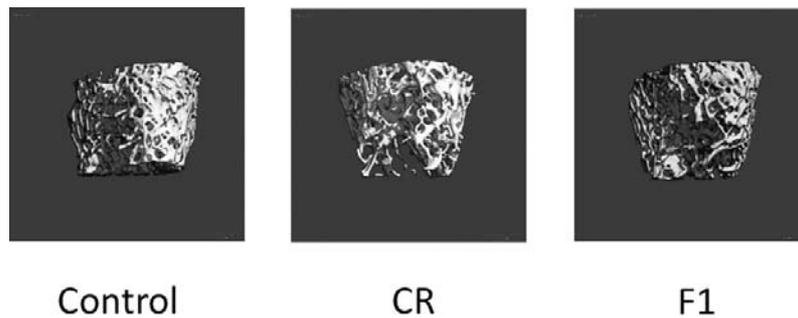


Figure 3: 3D-Micro-CT images of trabecular bone. 3D-Micro-CT images showed that control rats have more and thicker trabecular bone than CR and F1 groups

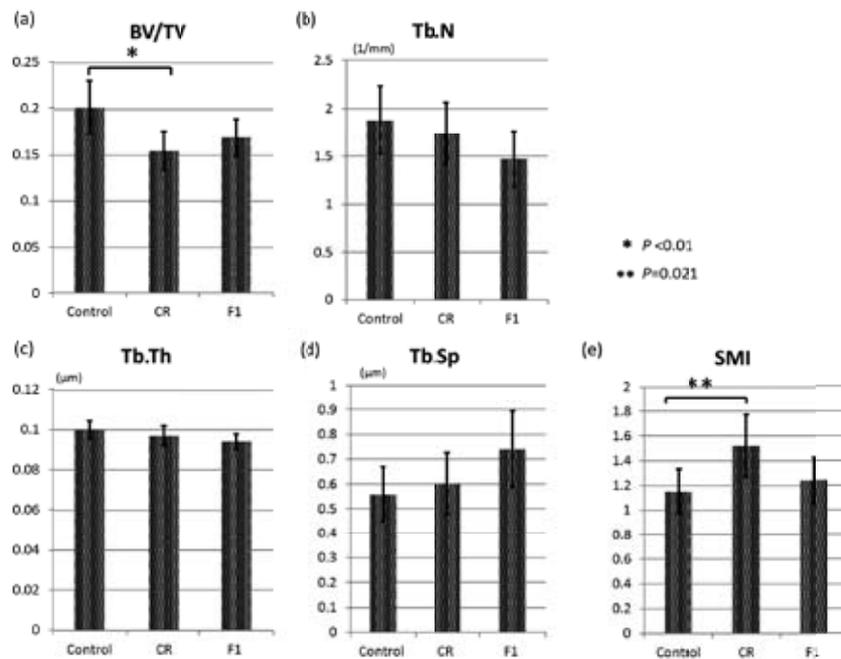


Figure 4: Parameters of trabecular bone microarchitecture with micro-CT analysis.

(a–e) The trabecular bone architecture, including the bone volume fraction (BV/TV), trabecular thickness (Tb.Th), trabecular number (Tb.N), trabecular separation (Tb.Sp) and structure model index (SMI), at the femoral condyle of rats measured with micro-CT.

(a) Mean BV/TV was significantly lower in CR group than Control group ($P < 0.01$).

(b–d) There was no significant difference in Tb.N, Tb.Th, and Tb.SP among three groups

(e) The mean SMI value in CR rats was marginally significant difference from that in control rats, ($P = 0.021$)

Discussion

Factors affecting bone metabolism, including hormones [13], neuronal [14] and mechanical stress [15], have been suggested. Because previous studies have reported that the GH-IGF-1 axis is suppressed by CR in experimental animals, we hypothesised that the suppression of this axis may be linked with changes in bone metabolism in CR rats. While there was no difference in mean serum IGF-1 concentration between CR and control groups, the current study showed significantly lower mean bone volume fraction and significantly higher SMI values in CR rats. Contrastingly, the mean serum IGF-1 levels in the F1 group were lower than those in controls; however, there was no significant difference in the mean bone volume fraction and SMI values between F1 and control rats. Because serum IGF-1 concentration and bone metabolism differed between CR and rats with GH-IGF-1 axis suppression (i.e. CR and F1 rats), it is possible that different pathways other than the GH-IGF-1 axis lead to the decreased bone volume and changes in bone structure seen in CR rats.

The GH-IGF-1 axis plays an important role in bone metabolism; the detailed mechanism of GH-IGF-1 axis effects on age-related changes in bone metabolism is not unclear. In liver-specific IGF-1-deficient mice, which exhibit a 75% reduction in serum IGF-1 levels, cortical bone volume is markedly reduced compared with that of normal mice, while cancellous bone volume was not significantly different [16,17]. Conversely, transgenic mice overexpressing osteoblast-specific IGF-1 show an increase in cancellous bone volume, but not in cortical bone volume [18]. The present study also shows the possibility that serum IGF-1 concentration does not affect the cancellous bone fraction.

Some studies have reported a significant association between body weight and bone volume. For example, low body weight caused by anorexia nervosa has been associated with reduced bone mass [19], while the body weight of C57BL/6 mice, those were the most widely used inbred strain mouse, has been associated with cortical bone mass [20]. Our study shows that mean body weight and bone volume fraction in CR rats were markedly lower than in controls. Although the mean body weight of CR rats was markedly heavier than that of F1 rats, there was no significant difference in BV/TV between them, even though BV/TV tended to be lower in CR rats. This result suggested that the reduction of cancellous bone volume was not caused by a reduction of body weight alone.

Because the reduced bone volume in CR rats was likely caused by a decline in bone turnover, namely osteoblastic

bone formation, we measured the mean serum BAP concentration. However, there was no significant difference in the concentration of this bone formation marker between any of the three groups. We believe that this is because we used 10-month-old rats that had already reached peak bone mass, thereby masking any differences between the three groups.

Bone remodelling functions are under cytokine, hormonal and neuronal control [14]. For example, leptin has been shown to control bone metabolism through the nervous system [21]. Moreover, depending on the effect of leptin in the hypothalamus, increases or decreases in orexigenic factors, such as neuropeptide Y or cocaine- and amphetamine-regulated transcripts, have also been shown to affect bone metabolism [14]. In the previous study of our group, it was suggested that plasma leptin concentration was highest in control, next in F1 rats, and lowest CR rats [9]. The changes of plasma leptin concentration were very similar to the changes of BV/TV in three groups. Therefore, it is possible that the may affect bone metabolism in CR rats.

Our cross-sectional study evaluating CR on bone metabolism in 10-month-old rats is limited by some facts. Firstly, we could not evaluate changes in bone metabolism as a function of age. Secondly, we did not evaluate cortical bone architecture, which could have altered our current results. Thirdly, the small number of mice in each groups may have prevented our making definitive conclusions.

Several studies have examined bone metabolism associated with CR or depressed IGF-1 levels in animal models. However, to the best of our knowledge, no report has yet experimentally compared bone metabolism associated with CR with that caused by GH-IGF-1 axis suppression in animals. In the present study, we compared cancellous bone metabolism in 10-month-old CR rats to that in F1 rats with a suppressed GH-IGF-1 axis. The results of our study suggest that changes in bone metabolism in CR and GH-IGF-1 axis-suppressed rats were dissimilar.

Conflict of Interests

The authors declare that there is no conflict of interest regarding the publication of this manuscript.

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