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ORIGINAL PAPER

Effects of hypergravity on gene levels in anti-gravity muscle and bone through the vestibular system in mice

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Abstract We recently reported that hypergravity with 3 *g* for 4 weeks affects muscle and bone through the vestibular system in mice. The purpose of this study was to investigate the effects of hypergravity with 2 *g*, which had no influence on circulating glucocorticoid level, on the gene levels in muscle and bone, as well as the roles of the vestibular system in those changes using vestibular lesioned (VL) mice. Hypergravity for 2 and 8 weeks or VL exerted little effects on the mRNA levels of muscle differentiation factors and myokines in the soleus muscle. Although hypergravity for 2 weeks significantly elevated alkaline phosphatase (ALP) and type I collagen mRNA levels in the tibia, VL significantly attenuated the levels of ALP mRNA enhanced by hypergravity. In conclusion, the present study suggests that a 2-*g* load for 2 weeks enhances osteoblast differentiation partly through the vestibular system in mice.

Keywords Gravity change · Muscle · Bone · Vestibular system

Introduction

Long-term bed rest and spaceflight induce muscle atrophy and bone loss [1–4]. Mechanical unloading markedly reduces muscle mass in the slow-twitch fiber dominant muscle such as the soleus muscle [5]. These findings suggest that mechanical stress plays a crucial role in maintaining muscle and bone, but its precise mechanisms by which mechanical stress regulates muscle and bone are not fully understood. The relationships between muscle and bone metabolism have been noted. A previous study showed that muscle atrophy precedes bone loss during mechanical unloading in mice [6]. Moreover, recovery from decreased muscle mass precedes amelioration of osteoporosis in astronauts after return to ground [7]. These findings suggest that a linkage between muscle and bone is involved in the effects of mechanical stress or gravity change on muscle and bone.

Skeletal myogenesis is regulated by myogenic regulatory factors including MyoD, Myf5, and myogenin [8]. Myosin heavy chains (MHC) are abundantly expressed in myotubes [9]. Bone mass is controlled by osteoblastic bone formation and osteoclastic bone resorption [10]. Osteoblasts are originally differentiated from mesenchymal stem

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cells accompanied with increased levels of Runx2, Osterix, alkaline phosphatase (ALP), type I collagen, and osteocalcin. Osteoclast differentiation is mainly regulated by the balance of receptor activator of nuclear factor-κB ligand (RANKL) and its decoy receptor osteoprotegerin (OPG) levels [11].

Organisms adapt to surrounding environmental changes through activation of various biological regulatory systems, such as sympathetic nervous system and the hypothalamic/pituitary/adrenal system [12]. However, excess stress hormones, glucocorticoids, and catecholamines cause dysregulation of physiological functions such as the cardiovascular system and metabolism. Glucocorticoid excess induces muscle atrophy through a decrease in muscle protein synthesis and an increase in muscle protein degradation [13]. Moreover, it induces osteoporosis mainly through suppression of osteoblastic bone formation [14]. Previous studies showed that gravity change induces stress response accompanied by elevated blood corticosterone levels in mice and rats [15, 16]. We recently reported that hypergravity with 3 *g* for 4 weeks increases muscle and bone mass through the vestibular system in mice. However, hypergravity with 3 *g* for 4 weeks did not affect the expression of osteoblast differentiation-related genes in tibia, and serum corticosterone levels were elevated in those mice [17]. These findings suggest a possibility that stress response might modulate muscle and bone during exposure to 3 *g* in mice.

A previous study revealed that exposure to 2 *g*, but not 3 *g*, for 3 weeks did not affect serum corticosterone levels in mice [16]. We therefore speculated that we could evaluate the effects of hypergravity on muscle and bone without the influences of glucocorticoid excess using the experiments with exposure to 2 *g*, but not 3 *g*, in mice. The purpose of this study was to examine the effects of 2 *g* load on the levels of myogenic and osteogenic differentiation genes in the soleus muscle and tibia of mice to clarify the mechanisms of gravity change-induced muscle and bone changes with less stress responses. Moreover, we investigated the roles of the vestibular system in the effects of 2 *g* load-induced muscle and bone changes using vestibular lesioned (VL) mice.

Materials and methods

Animal experiments

Male C57BL/6J, 6-week-old mice ($n = 54$), were purchased from Chubu Kagaku Shizai (Nagoya, Japan), and randomly divided into two groups: sham ($n = 28$) or VL ($n = 26$) surgery. Sham or VL surgery was bilaterally performed on all mice at the same time under 2% isoflurane anesthesia, according to the method described previously [17–21]. Buprenorphine (3 μg/kg, Lepetan, Otsuka, Tokyo, Japan) and penicillin G potassium (3000 U/kg, Meiji Seika Pharma, Tokyo, Japan) were subcutaneously administered to all mice for pain relief and prevention of infections after the surgery. The evaluation of VL was performed by swimming test and mid-air righting reflex test, as described previously [17–20]. After 2 weeks as a recovery period, the mice were divided into four groups: 1 *g*/sham ($n = 14$), 2 *g*/sham ($n = 14$), 1 *g*/VL ($n = 13$), and 2 *g*/VL ($n = 13$). Mice were exposed to 2-*g* environments induced by centrifugation in a custom-made, gondola-type rotating box for 2 and 8 weeks, as described previously [17]. All mice were fed ad libitum with food and water. The room temperature was kept at 24 ± 1 °C with a 12 h:12 h light/dark cycle. The experiments were performed according to the guidelines of the National Institutes of Health and the "Guiding Principles for Care and Use of Animals in the Field of Physiological Science" set by the Physiological Society of Japan. The experiments were approved by the Animal Research Committees of Gifu University (27–79) and the Japan Aerospace Exploration Agency (015–008).

Quantitative real-time PCR

Total RNA was isolated from the soleus and tibia using an RNeasy Mini Kit (Qiagen, Hilden, Germany). Reverse transcription was performed using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster, CA, USA). Quantitative real-time PCR was performed using a SYBR Green Master Mix and an ABI StepOne Real-Time PCR System to analyze muscle differentiation-related genes (MyoD, Myf5, myogenin, and MHC-I), muscle protein degradation-related genes (atrogin-1 and MuRF1), osteogenic genes (Runx2, Osterix, ALP, type I collagen and osteocalcin), bone resorption-related genes [RANKL, OPG and receptor activator of nuclear factor-κB (RANK)] and humoral factor genes linking muscle to bone [insulin-like growth factor-1 (IGF-1), fibroblast growth factor 2 (FGF2), transforming growth factor-β (TGF-β), myostatin, activin A, follistatin, fibronectin type III domain-containing 5 (FNDC5), interleukin-6 (IL-6), osteoglycin and family with sequence similarity 5, member C (FAM5C)], as previously described [22]. Each PCR primer is shown in Table S1. The specific mRNA amplification of the target was determined as the Ct value, which was followed by normalization with the glyceraldehyde-3-phosphate dehydrogenase level.

Blood chemistry

Serum corticosterone levels were measured using OCTAEIA Corticosterone enzymeimmunoassay kit

(Immunodiagnostic Systems, Fountain Hills, AZ, USA), as described previously [17].

Statistical analysis

Data are expressed as the mean ± the standard error of the mean (SEM). Data were analyzed using two-way ANOVA followed by the Tukey–Kramer test for multiple comparisons. The significance level was set at $P < 0.05$. All statistical analyses were performed using GraphPad PRISM 7.00 software.

Results

Effects of hypergravity and VL on serum corticosterone levels in mice

We examined serum corticosterone levels in mice after exposure to 2 *g* for 2 and 8 weeks in order to clarify whether 2-*g* load induces stress response in the present study. There are no significant differences in serum corticosterone levels between 1- and 2-*g* mice after 2 or 8 weeks (Fig. 1a, b). VL did not affect serum corticosterone levels in 1-*g* and 2-*g* mice (Fig. 1a, b). Although VL seemed to elevate serum corticosterone levels in mice 2 weeks after exposure to 1 or 2 *g*, there were no significant differences between sham-1 *g* and VL-1 *g* mice ($P = 0.109$) or between sham-2 *g* and VL-2 *g* mice ($P = 0.131$).

Effects of hypergravity and VL on muscle differentiation-related gene levels in the soleus muscle of mice

Our previous study showed that exposure to 3 *g* for 4 weeks significantly elevated the mRNA levels of muscle differentiation factors, including MyoD and myogenin, in the soleus muscle of mice through the vestibular system [17]. We therefore examined the effects of 2-*g* load and VL on the gene levels of muscle differentiation factors in mice. Exposure to 2 *g* for 2 or 8 weeks did not affect the mRNA levels of Myf5, myogenin, and MHC-I in the soleus muscle of mice compared to those of 1-*g* mice (Fig. 2a, b). On the other hand, exposure to 2 *g* for 8 weeks significantly reduced MyoD mRNA levels in sham mice compared to those of 1-*g* sham mice (Fig. 2b). VL did not affect the mRNA levels of MyoD, Myf5, myogenin, and MHC-I in the soleus muscle of 1- and 2-*g* mice (Fig. 2a, b). Moreover, exposure to 2 *g* for 2 or 8 weeks or VL did not affect the mRNA levels of atrogin-1 and MuRF1, muscle protein degradation-related genes, in the soleus muscle of mice (Fig. 2a, b).

Effects of hypergravity and VL on osteogenic and bone resorption-related gene levels in the tibia of mice

We next examined the mRNA levels of osteogenic genes and RANKL/OPG in the tibia of mice. Exposure to 2 *g* for 2 weeks significantly elevated ALP and type I collagen mRNA levels in the tibia of mice, compared to 1-*g* mice (Fig. 3a). Moreover, the mRNA levels of Runx2, Osterix, and osteocalcin in the tibia seem to be increased by 2-*g* exposure, although it did not reach a statistically significant level. In VL mice, the mRNA levels of ALP and Osterix in the tibia were significantly reduced by 2 *g* for 2 weeks. The mRNA levels of type I collagen and osteocalcin in the tibia of VL mice also seem to be reduced by 2 *g* for 2 weeks, but it did not reach statistically significant level. On the other hand, exposure to 2 *g* for 8 weeks or VL did not affect the mRNA levels of Runx2, Osterix, ALP, type I collagen and osteocalcin in the tibia, compared to 1-*g* sham mice (Fig. 3b). Since osteoclast differentiation

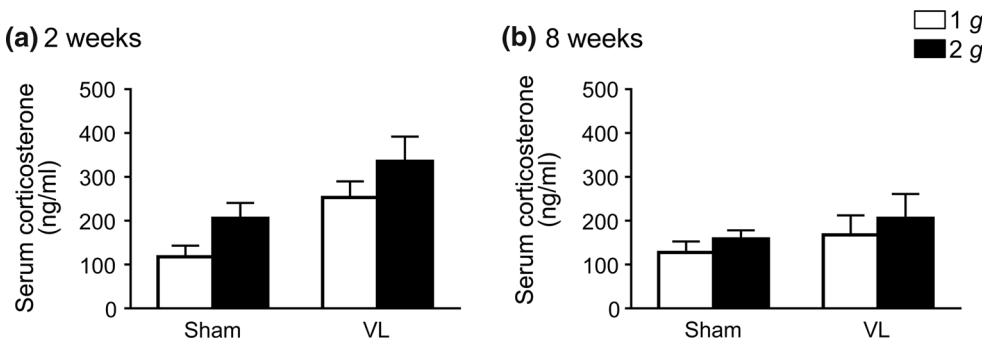

Fig. 1 Effects of hypergravity and vestibular lesion (VL) on serum corticosterone levels in mice. Blood samples were collected from mice treated with VL or sham surgery after exposure to 1 *g* or 2 *g* for 2 weeks (**a**) and 8 weeks (**b**). Then, serum corticosterone levels were measured. Data represent the mean ± SEM of six mice in each group (**a**) as well as 8 (1 *g*-sham, 2 *g*-sham) and 7 (1 *g*-VL, 2 *g*-VL) mice (**b**). *Open bar* and *filled bar* represent 1 and 2 *g*, respectively

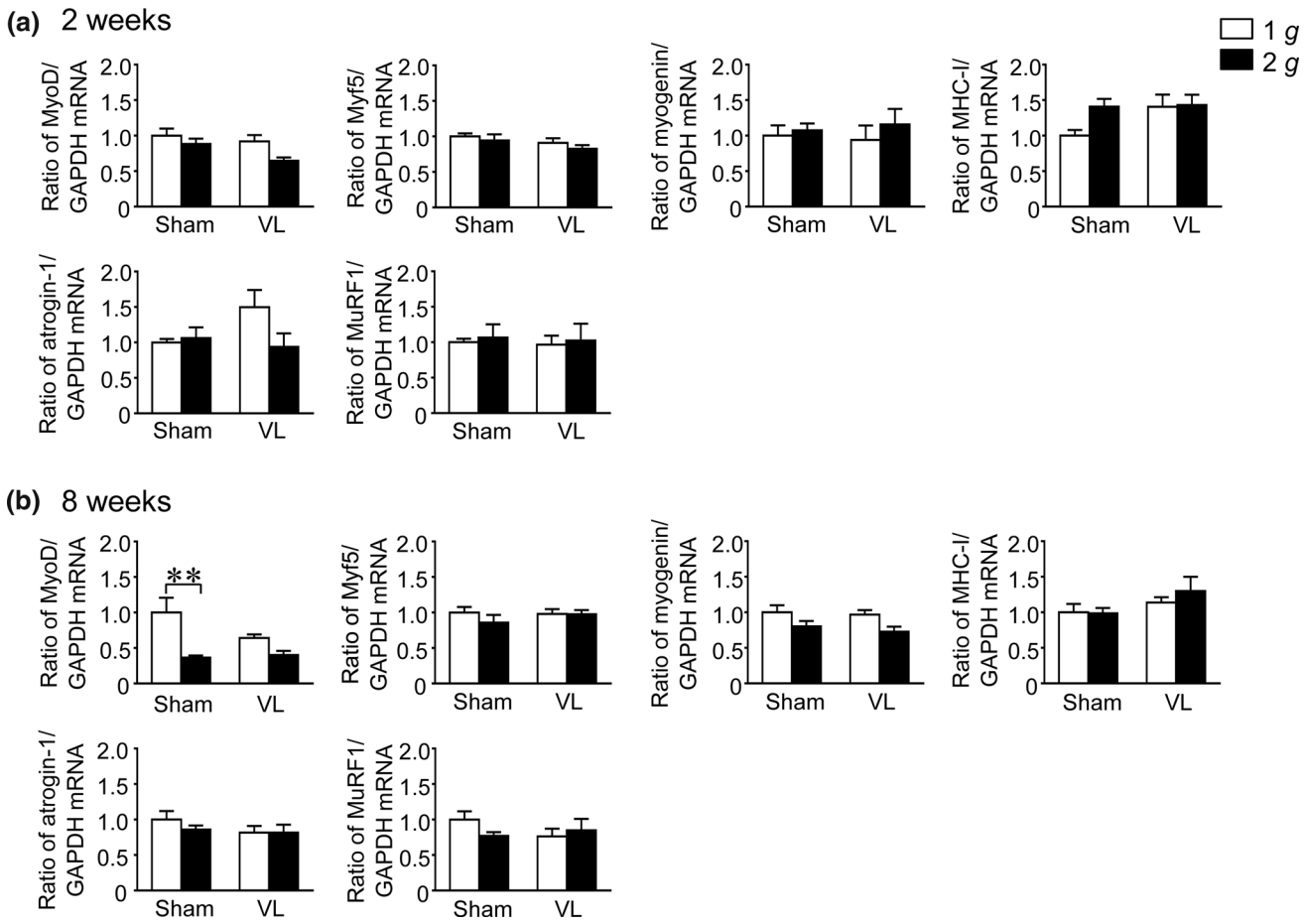

Fig. 2 Effects of hypergravity and VL on the expression of myogenic differentiation genes in the soleus muscle of mice. Total RNA was extracted from the soleus muscle of mice treated with VL or sham surgery after exposure to 1 or 2 *g* for 2 weeks (**a**) and 8 weeks (**b**). Then, the level of MyoD, Myf5, myogenin, myosin heavy chain-I (MHC-I), atrogin-1, MuRF1 or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was assessed by real-time PCR analysis. Data are expressed relative to the levels of GAPDH. The data represent the mean ± SEM of 6 (**a**) and 4 (**b**) tissues in each group. $^{**}P < 0.01$

is regulated by the balance of RANKL and OPG, we next examined the effects of 2-*g* load and VL on RANKL, OPG and RANK mRNA levels in the tibia. Exposure to 2 *g* for 2 or 8 weeks did not affect the mRNA levels of RANKL, OPG and RANK as well as the ratio of RANKL to OPG in the tibia of mice (Fig. 4a, b).

Effects of hypergravity and VL on the gene levels of humoral factors linking muscle to bone in the soleus muscle of mice

Myokines, such as IGF-1, FGF2, TGF-β, myostatin, activin A, follistatin, irisin, IL-6, osteoglycin and FAM5C, are included in humoral factors linking muscle to bone [1]. We therefore examined the effects of 2-*g* load or VL on gene levels of humoral factors linking muscle to bone in the soleus muscle of mice. Although exposure to 2 *g* for 2 weeks significantly elevated TGF-β mRNA levels in the soleus muscle of mice exposed to 2 *g* for 2 weeks compared to those of 1-*g* mice, VL did not affect TGF-β mRNA levels enhanced by exposure to 2 *g* for 2 weeks (Table 1). Exposure to 2 *g* or VL did not affect the mRNA levels of IGF-1, FGF2, myostatin, activin A, follistatin, FNDC5, IL-6, osteoglycin, and FAM5C in the soleus muscle of mice after 2 and 8 weeks (Table 1).

Discussion

In the present study, we showed that VL blunted the 2 *g* for 2 weeks-enhanced expression of osteogenic genes, such as ALP and Osterix, in the bone tissues, although hypergravity or VL exerted little effect on the expression of antigravity muscle genes and RANKL/OPG in the bone tissues as well as circulating glucocorticoid levels.

Our previous study revealed that hypergravity with 3-*g* load for 4 weeks did not affect the expression of Runx2, Osterix, ALP, type I collagen, and osteocalcin in

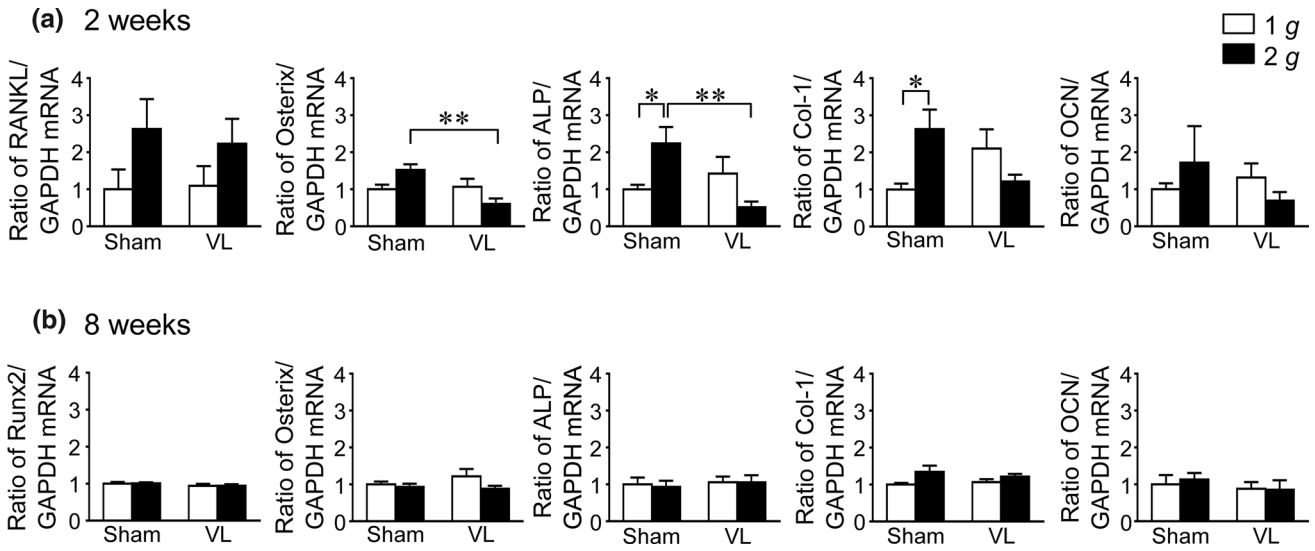

Fig. 3 Effects of hypergravity and VL on the expression of osteogenic differentiation genes in the tibia of mice. Total RNA was extracted from the tibia of mice treated with VL or sham surgery after exposure to 1 or 2 *g* for 2 (**a**) and 8 weeks (**b**). Then, the mRNA level of Runx2, Osterix, alkaline phosphatase (ALP), type I collagen (Col-1), osteocalcin (OCN) or GAPDH was assessed by real-time PCR analysis. Data are expressed relative to the levels of GAPDH. The data represent the mean ± SEM of 6 (**a**) and 4 (**b**) tissues in each group. $^{**}P < 0.01$, $^{*}P < 0.05$

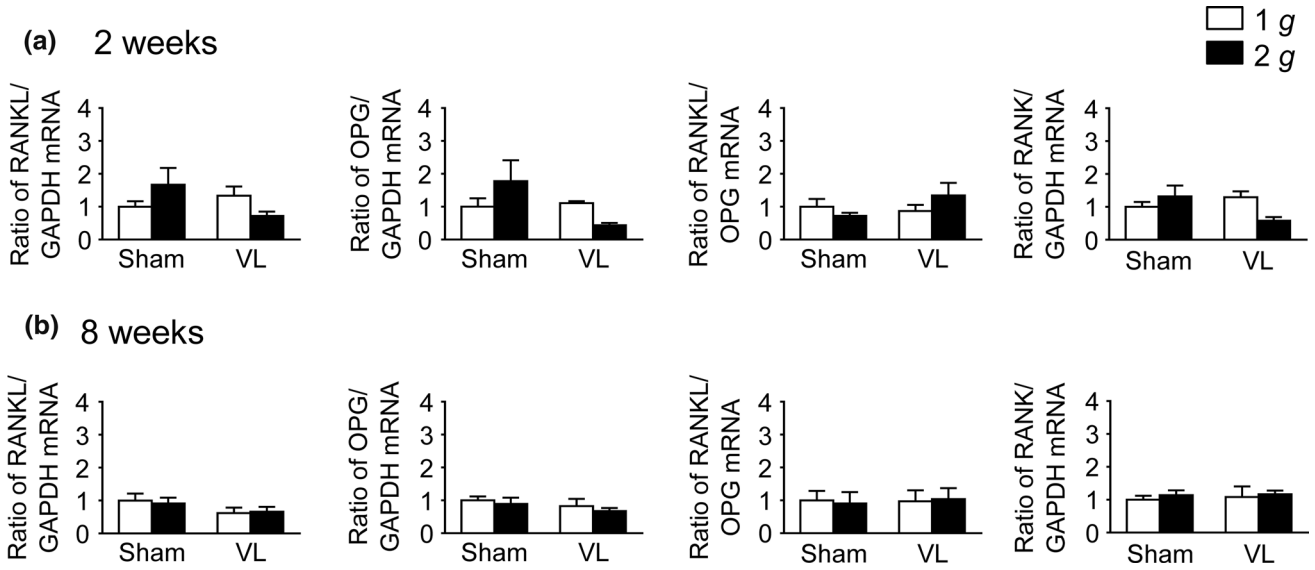

Fig. 4 Effects of hypergravity and VL on the expression of receptor activator of nuclear factor-κB ligand (RANKL), osteoprotegerin (OPG) and receptor activator of nuclear factor-κB (RANK) in the tibia of mice. Total RNA was extracted from the tibia of mice treated with VL or sham surgery after exposure to 1 *g* or 2 *g* for 2 (**a**) and 8 weeks (**b**). Then, the mRNA level of RANKL, OPG, RANK and GAPDH was assessed by real-time PCR analysis. Data are expressed relative to the levels of GAPDH and ratio of RANKL to OPG mRNA levels. The data represent the mean ± SEM of 6 (**a**) and 4 (**b**) tissues in each group

the tibia of mice [17]. However, our present study showed that hypergravity with 2-*g* load for 2 weeks significantly elevated the expression of ALP and type I collagen in the tibia of mice, and that it seemed to enhance the expression of Runx2, Osterix, and osteocalcin. Gnyubkin et al. [23] reported that chronic hypergravity with 2-*g* load induces beneficial effects, such as stimulation of osteoid deposition and angiogenesis, but that chronic 3-*g* load induces deleterious response on bone metabolism in mice. These findings suggest that the bone changes induced by hypergravity are dependent on the gravity levels. In our previous study, hypergravity with 3-*g* load elevated serum glucocorticoid levels in those mice [17]. On the other hand, exposure to 2 *g* for 2 weeks did not affect serum glucocorticoid levels in mice in the present study, which were compatible with a previous study indicating that 3-*g* but not

Table 1 Effects of hypergravity and vestibular lesion (VL) on the mRNA levels of humoral factors linking muscle to bone in the soleus muscle

Gene	2 weeks				8 weeks			
	Sham-1 *g*	Sham-2 *g*	VL-1 *g*	VL-2 *g*	Sham-1 *g*	Sham-2 *g*	VL-1 *g*	VL-2 *g*
IGF-1	1.00 ± 0.11	1.43 ± 0.11	1.07 ± 0.13	1.21 ± 0.16	1.00 ± 0.20	1.25 ± 0.18	1.51 ± 0.23	1.47 ± 0.22
FGF2	1.00 ± 0.16	1.02 ± 0.08	1.30 ± 0.26	1.07 ± 0.10	1.00 ± 0.14	1.07 ± 0.11	0.84 ± 0.05	1.31 ± 0.32
TGF-β1	1.00 ± 0.11	1.66 ± 0.14**	0.80 ± 0.13	1.24 ± 0.14	1.00 ± 0.23	0.83 ± 0.09	0.67 ± 0.07	1.16 ± 0.13
MSTN	1.00 ± 0.28	0.92 ± 0.32	0.76 ± 0.24	1.12 ± 0.51	1.00 ± 0.17	0.77 ± 0.19	0.58 ± 0.25	0.82 ± 0.29
Act A	1.00 ± 0.13	0.87 ± 0.05	0.95 ± 0.14	0.89 ± 0.05	1.00 ± 0.30	1.03 ± 0.09	1.42 ± 0.36	1.16 ± 0.25
FST	1.00 ± 0.12	1.08 ± 0.08	1.21 ± 0.04	1.13 ± 0.07	1.00 ± 0.27	0.75 ± 0.08	1.19 ± 0.18	0.94 ± 0.16
FNDC5	1.00 ± 0.09	1.27 ± 0.07	0.86 ± 0.12	0.99 ± 0.16	1.00 ± 0.09	1.22 ± 0.09	1.06 ± 0.15	1.28 ± 0.18
IL-6	1.00 ± 0.10	1.28 ± 0.25	1.23 ± 0.10	1.14 ± 0.13	1.00 ± 0.20	2.50 ± 0.71	2.28 ± 0.40	1.61 ± 0.61
OGN	1.00 ± 0.12	1.20 ± 0.11	1.12 ± 0.08	1.21 ± 0.38	1.00 ± 0.09	0.77 ± 0.15	0.96 ± 0.08	0.88 ± 0.12
FAM5C	1.00 ± 0.10	0.72 ± 0.08	1.08 ± 0.08	0.93 ± 0.09	1.00 ± 0.70	0.67 ± 0.09	1.13 ± 0.37	0.89 ± 0.35

Total RNA was extracted from the soleus muscle of mice treated with VL or sham surgery after exposure to 1 or 2 *g* for 2 and 8 weeks. Then, the level of insulin-like growth factor-1 (IGF-1), fibroblast growth factor 2 (FGF2), transforming growth factor-β1 (TGF-β1), myostatin (MSTN), activin A (Act A), follistatin (FST), fibronectin type III domain-containing 5 (FNDC5), interleukin-6 (IL-6), osteoglycin (OGN), family with sequence similarity 5, member C (FAM5C) or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was assessed by real-time PCR analysis. Data are expressed relative to the levels of GAPDH. The data represent the mean ± SEM of 6 (2 weeks) and 4 (8 weeks) tissues in each group.

** $P < 0.01$. vs. sham-1 *g*

2-*g* load for 3 weeks elevates levels of circulating glucocorticoid in mice [16]. Since excess glucocorticoid suppresses osteoblast differentiation, then leading to osteoporosis [14, 24], our findings suggest the possibility that stress response might blunt osteoblast differentiation enhanced by hypergravity during gravitational load of 3 *g*.

Gravity change is sensed by the vestibular system and its stimulation is transmitted to the vestibular nuclei, connecting to hypothalamus, sympathetic nervous system and skeletal muscle [25, 26]. Our previous study suggests that plastic change of the vestibular system contributes to orthostatic intolerance in astronauts after long-term spaceflight [27]. Moreover, we previously reported that 3-*g* load for 4 weeks affects muscle and bone through the vestibular system and subsequently through the sympathetic nervous system in mice [17, 19, 20]. These findings suggest that the vestibular system plays a crucial role in the responses to gravity change. In the present study, VL significantly blunted the expression of ALP and Osterix enhanced by 2-*g* load for 2 weeks and seemed to reduce the expression of type I collagen and osteocalcin during 2-*g* load for 2 weeks in mice, although 2-*g* load for 2 and 8 weeks or VL did not affect the expression of RANKL and OPG as well as the ratio of RANKL/OPG. These results suggest that hypergravity with 2-*g* load for 2 weeks facilitates osteoblast differentiation through the vestibular system in mice. Moreover, the effects of hypergravity with 2-*g* load for 2 weeks on bone are mainly on osteoblastic bone formation, but not RANKL-mediated bone resorption in mice.

The mechanisms by which hypergravity with 2 *g* for 8 weeks did not affect levels of osteogenic differentiation genes still remain unknown. Ishizawa et al. [28] revealed that the changes of vitamin D 24-hydroxylase and 1α-hydroxylase gene levels after exposure to 2 *g* for 2 days were diminished after exposure to 2 *g* for 14 days in the kidney of mice. Moreover, Tateishi et al. [29] showed that gene expression of Aire and RANK were increased in mature medullary thymic epithelial cells derived from mice after exposure to 2 *g* for 3 days, and subsequently reduced in those after exposure to 2 *g* load for 14 days. Taking into account the finding that the vestibular system has a high plasticity [30], we speculated that the effects of hypergravity with 2 *g* for 8 weeks on tibia gene expression through the vestibular system might be diminished in mice for adaptation to hypergravity environment.

We showed that hypergravity with 2 *g* load did not affect the expression of MyoD, Myf5, myogenin, and MHC-I as well as atrogin-1 and MuRF1 in the soleus muscle of mice, except for the effects of 2-*g* load for 8 weeks on the levels of MyoD mRNA in the present study. Our previous study showed that hypergravity with 3-*g* load for 4 weeks significantly elevates levels of MyoD and myogenin mRNA in the soleus muscle of mice [17]. These findings suggest that 2-*g* load might be insufficient for the acceleration of myogenic differentiation, compared to 3-*g* load in mice. The mechanisms by which 2-*g* load for 8 weeks reduces MyoD mRNA levels in the soleus muscle still remains unknown. Casey et al. [31] revealed that hypergravity modulates the expression of DNA methyltransferases in rats. Moreover, Montesano et al. [32] reported that the DNA demethylation agent 5-azacytidine enhanced MyoD levels in mouse myoblastic C2C12 cells. Taken together, we speculated that epigenetic changes

might be involved in the effects of 2-*g* load for 8 weeks on MyoD mRNA levels. Moreover, we cannot exclude the possibility that the effects of VL itself concealed those of exposure to 2-*g* load on MyoD mRNA levels in the soleus muscle of mice, although VL did not significantly affect MyoD mRNA levels. It has been previously reported that MHC isoforms did not change in the soleus muscle of rats after exposure to 2 *g* for 2 weeks, although 2-*g* load for 8 weeks induces a shift of MHC isoforms from slow-twitch to fast-twitch [33]. We therefore could not rule out the possibility that more than 8 weeks are required for facilitation of myogenic differentiation by 2-*g* load in the soleus muscle of mice.

Interaction between muscle and bone has been recently recognized [1, 34]. Skeletal muscle influences bone metabolism by production of local and humoral factors. Exercise and mechanical stress may play crucial roles in the regulation of humoral factors linking muscle to bone [34, 35]. Our recent study showed that hypergravity with 3-*g* load for 4 weeks increases levels of follistatin in the circulating blood and soleus muscle of mice through the vestibular system [19]. In that study, follistatin released from the skeletal muscle might affect bone metabolism by suppressing osteoclast formation and facilitating differentiation of mesenchymal stem cells into osteoblastic cells in an endocrine manner in mice [19]. Taken together, gravity change might affect bone metabolism by myokine release from the skeletal muscle through the vestibular system. In the present study, 2-*g* load for 2 weeks enhanced osteogenic factors partly through the vestibular system in the tibia of mice. However, hypergravity with 2-*g* load for 2 or 8 weeks did not affect the expression of humoral factors linking muscle to bone, except for TGF-β, in the soleus muscle of mice. These findings suggest that 2-*g* load for 2 weeks affects osteoblasts through the vestibular system independently of muscle-derived humoral factors in mice. Further studies are necessary to clarify the detailed mechanism of the vestibular system-mediated osteoblastic differentiation and roles of TGF-β released from muscle during gravity change.

In conclusion, we showed that hypergravity with 2-*g* load for 2 weeks enhances osteoblastic differentiation partly through the vestibular system in mice. The present study suggests that the vestibular system contributes to adaptive responses in bone tissues by affecting osteoblast differentiation, but not muscle differentiation, during 2-*g* load without the influences on circulating glucocorticoid levels in mice.

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Author contributions NK, HM, and HK contributed to the conception and design of the research. NK, HM, KN, KO, and KT performed the experiments. NK and KN analyzed the data. NK, HM and HK interpreted the results of the experiments. NK and HK prepared the figures. NK and HK drafted the manuscript. All authors approved final version of manuscript.

Compliance with ethical standards

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Conflict of interest The authors declare that they have no conflicts of interest.

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. This article does not contain any studies with human participants performed by any of the authors.

References

1. Kawao N, Kaji H (2015) Interactions between muscle tissues and bone metabolism. J Cell Biochem 116:687–695
2. Cohen S, Nathan JA, Goldberg AL (2015) Muscle wasting in disease: molecular mechanisms and promising therapies. Nat Rev Drug Discov 14:58–74
3. Stein TP (2013) Weight, muscle and bone loss during space flight: another perspective. Eur J Appl Physiol 113:2171–2181
4. Tanaka K, Nishimura N, Kawai Y (2017) Adaptation to microgravity, deconditioning, and countermeasures. J Physiol Sci 67:271–281
5. Fitts RH, Trappe SW, Costill DL, Gallagher PM, Creer AC, Colloton PA, Peters JR, Romatowski JG, Bain JL, Riley DA (2010) Prolonged space flight-induced alterations in the structure and function of human skeletal muscle fibres. J Physiol 588:3567–3592
6. Lloyd SA, Lang CH, Zhang Y, Paul EM, Laufenberg LJ, Lewis GS, Donahue HJ (2014) Interdependence of muscle atrophy and bone loss induced by mechanical unloading. J Bone Miner Res 29:1118–1130
7. Keyak JH, Koyama AK, LeBlanc A, Lu Y, Lang TF (2009) Reduction in proximal femoral strength due to long-duration spaceflight. Bone 44:449–453
8. Endo T (2015) Molecular mechanisms of skeletal muscle development, regeneration, and osteogenic conversion. Bone 80:2–13
9. Schiaffino S, Reggiani C (2011) Fiber types in mammalian skeletal muscles. Physiol Rev 91:1447–1531
10. Komori T (2015) Animal models for osteoporosis. Eur J Pharmacol 759:287–294
11. Henriksen K, Bollerslev J, Everts V, Karsdal MA (2011) Osteoclast activity and subtypes as a function of physiology and pathology—implications for future treatments of osteoporosis. Endocr Rev 32:31–63

12. Elenkov IJ, Chrousos GP (2006) Stress system—organization, physiology and immunoregulation. Neuroimmunomodulation 13:257–267
13. Braun TP, Marks DL (2015) The regulation of muscle mass by endogenous glucocorticoids. Front Physiol 6:12
14. Canalis E, Mazziotti G, Giustina A, Bilezikian JP (2007) Glucocorticoid-induced osteoporosis: pathophysiology and therapy. Osteoporos Int 18:1319–1328
15. Abe C, Ueta Y, Morita H (2013) Exposure to hypergravity during the preweaning but not postweaning period reduces vestibular-related stress responses in rats. J Appl Physiol (1985) 115:1082–1087
16. Gueguinou N, Bojados M, Jamon M, Derradji H, Baatout S, Tschirhart E, Frippiat JP, Legrand-Frossi C (2012) Stress response and humoral immune system alterations related to chronic hypergravity in mice. Psychoneuroendocrinology 37:137–147
17. Kawao N, Morita H, Obata K, Tamura Y, Okumoto K, Kaji H (2016) The vestibular system is critical for the changes in muscle and bone induced by hypergravity in mice. Physiol Rep 4:e12979
18. Morita H, Obata K, Abe C, Shiba D, Shirakawa M, Kudo T, Takahashi S (2015) Feasibility of a short-arm centrifuge for mouse hypergravity experiments. PLoS One 10:e0133981
19. Kawao N, Morita H, Obata K, Tatsumi K, Kaji H (2017) Role of follistatin in muscle and bone alterations induced by gravity change in mice. J Cell Physiol. doi:10.1002/jcp.25986
20. Shimoide T, Kawao N, Tamura Y, Morita H, Kaji H (2016) Novel roles of FKBP5 in muscle alteration induced by gravity change in mice. Biochem Biophys Res Commun 479:602–606
21. Morita H, Yamaguchi A, Shiba D, Shirakawa M, Takahashi S (2017) Impact of a simulated gravity load for atmospheric reentry, 10 g for 2 min, on conscious mice. J Physiol Sci 67:531–537
22. Kawao N, Tamura Y, Okumoto K, Yano M, Okada K, Matsuo O, Kaji H (2013) Plasminogen plays a crucial role in bone repair. J Bone Miner Res 28:1561–1574
23. Gnyubkin V, Guignandon A, Laroche N, Vanden-Bossche A, Normand M, Lafage-Proust MH, Vico L (2015) Effects of chronic hypergravity: from adaptive to deleterious responses in growing mouse skeleton. J Appl Physiol (1985) 119:908–917
24. Olkku A, Mahonen A (2009) Calreticulin mediated glucocorticoid receptor export is involved in beta-catenin translocation and Wnt signalling inhibition in human osteoblastic cells. Bone 44:555–565
25. Yates BJ, Bolton PS, Macefield VG (2014) Vestibulo-sympathetic responses. Compr Physiol 4:851–887
26. Markia B, Kovacs ZI, Palkovits M (2008) Projections from the vestibular nuclei to the hypothalamic paraventricular nucleus: morphological evidence for the existence of a vestibular stress pathway in the rat brain. Brain Struct Funct 213:239–245
27. Morita H, Abe C, Tanaka K (2016) Long-term exposure to microgravity impairs vestibulo-cardiovascular reflex. Sci Rep 6:33405
28. Ishizawa M, Iwasaki K, Kato S, Makishima M (2009) Hypergravity modulates vitamin D receptor target gene mRNA expression in mice. Am J Physiol Endocrinol Metab 297:E728–E734
29. Tateishi R, Akiyama N, Miyauchi M, Yoshinaga R, Sasanuma H, Kudo T, Shimbo M, Shinohara M, Obata K, Inoue J, Shirakawa M, Shiba D, Asahara H, Yoshida N, Takahashi S, Morita H, Akiyama T (2015) Hypergravity provokes a temporary reduction in CD4+ CD8+ thymocyte number and a persistent decrease in medullary thymic epithelial cell frequency in mice. PLoS One 10:e0141650
30. Clarke AH, Grigull J, Mueller R, Scherer H (2000) The three-dimensional vestibulo-ocular reflex during prolonged microgravity. Exp Brain Res 134:322–334
31. Casey T, Patel OV, Plaut K (2015) Transcriptomes reveal alterations in gravity impact circadian clocks and activate mechanotransduction pathways with adaptation through epigenetic change. Physiol Genom 47:113–128
32. Montesano A, Luzi L, Senesi P, Terruzzi I (2013) Modulation of cell cycle progression by 5-azacytidine is associated with early myogenesis induction in murine myoblasts. Int J Biol Sci 9:391–402
33. Fuller PM, Baldwin KM, Fuller CA (2006) Parallel and divergent adaptations of rat soleus and plantaris to chronic exercise and hypergravity. Am J Physiol Regul Integr Comp Physiol 290:R442–R448
34. Kaji H (2016) Effects of myokines on bone. Bonekey Rep 5:826
35. Laurent MR, Dubois V, Claessens F, Verschueren SM, Vanderschueren D, Gielen E, Jardi F (2016) Muscle–bone interactions: from experimental models to the clinic? A critical update. Mol Cell Endocrinol 432:14–36