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Research Article

Knockouts of Se-glutathione peroxidase-1 and Cu,Zn superoxide dismutase exert different impacts on femoral mechanical performance of growing mice

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The objective of this study was to determine the impact of knockout of Cu,Zn-superoxide dismutase (SOD1) and Se-glutathione peroxidase-1 (GPX1) on murine bone biomechanical properties. Femora samples were collected from wild-type (WT), SOD1-knockout [SOD1(−/−)] and GPX1-knockout [GPX1(−/−)] female mice (9-wk old, $n = 7-8$ *per* genotype) to assay for bone enzyme activities and mechanical properties in three point bending. Prior to testing, all mice were fed a torula yeast diet supplemented with 0.4 mg Se/kg as sodium selenite. Compared with the WT mice, SOD1(−/−) mice displayed a series of reductions ($p < 0.05$): 24% in body mass, 8% in femoral length, 43% in femoral structural strength, and 32% in bending stiffness. When differences in body size were accounted for, femoral failure moment in SOD1(−/−) mice remained lower ($p < 0.05$) than that of WT. Femoral tartrate resistant acid phosphatase activity in SOD1(−/−) was 47% greater ($p < 0.05$) than the WT. In contrast, GPX1(−/−) mice showed no significant differences in femoral mechanical properties from those of WT mice. In conclusion, knockout of SOD1 exerted a greater impact on femoral mechanical characteristics than that of GPX1 in growing mice.

Keywords: Antioxidant enzyme / Biomechanics / Bone / Mouse / Nutrition

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1 Introduction

Reactive oxygen species (ROS) such as superoxide (O_2^-) and hydrogen peroxide (H_2O_2) have been implicated in bone metabolism by mediating the process of osteoclastic resorption [1–4] and osteoblastic differentiation [5]. Excessive ROS may cause apoptosis of osteoblasts [6] and consequently bone metabolic disorders [7, 8]. Thus, regulations of intracellular ROS concentrations by antioxidant enzyme systems are critical for skeletal development and integrity.

Superoxide dismutases (SOD) and glutathione peroxidases are two major antioxidant enzyme families in mammals for coping with ROS-mediated oxidative stress. The SOD enzymes catalyze the dismutation of superoxide anion into H_2O_2 [9], whereas the GPX enzymes subsequently reduce H_2O_2 into water [10]. Specifically, Cu,Zn-superoxide dismutase (SOD1) is expressed in cytosol and intermembrane of mitochondria [11], and has been shown to inhibit the superoxide-stimulated osteoclastic bone resorption *in vitro* [12]. Cellular Se-glutathione peroxidase-1 (GPX1) is the first identified and most abundant selenoprotein [10]. There are increasing evidence to show a link between selenium and bone integrity in both humans and animals. While selenium is not the only etiological factor in Kashin-Beck Disease, its deficiency clearly contributes to its incidence [13].

A correlation between selenium intake and risk of hip fracture has been reported [14]. While selenium (along with iodide) deficiency decreased growth of tibia and cartilage and expression of type X collagen in a rat model of Kashin-Beck osteoarthropathy [15], supplemental selenium, along with vitamins E and C, restored bone structural alteration in a rabbit model of osteoporosis [16]. In these cases, selenium has been speculated to exert its impacts by influencing antioxidant defenses in bone. Previous studies in soft tissues

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Abbreviations: GPX1, Se-glutathione peroxidase-1; **ROS,** reactive oxygen species; **SOD1,** Cu,Zn-superoxide dismutase; **SOD1(−/−),** SOD1 knockout; **WT,** wild-type

indicate that GPX1 is the metabolic mediator of body Se in protecting against acute oxidative stress [17, 18]. In fact, GPX1 has been considered a predominantly expressed H_2O_2 scavenger in osteoclasts [19]. Along with SOD1, GPX1 may affect physiological balance of ROS in the bone microenvironment [20]. However, current literature on the interaction between antioxidant systems and ROS in bone metabolism [21–23] has been largely derived from *in vitro* studies or short-term supplement experiments [24]. Due to the limitations of conventional animal models, the specific roles of individual antioxidant enzymes such as SOD1 and GPX1 in bone growth and development under physiological conditions have not been well studied.

We have successfully applied SOD1-knockout [SOD1(−/−)] and GPX1 knockout [GPX1(−/−)] mice to elucidate the *in vivo* antioxidant role in oxidative stress targeting internal soft tissues [25]. Because our results have shown that these models are specific and physiological, they should be also an excellent tool for assessing the *in vivo* role of SOD1 and GPX1 in the murine skeleton. In bone metabolism, alkaline phosphatase and tartrate resistant acid phosphatase have been considered sensitive biochemical indicators of osteoblastic [26–27] and osteoclastic activities [28–30], respectively. Bone mechanical properties in three-point bending provide critical information on bone load-bearing ability beyond bone mass or mineral density for predicting fracture risk [21, 31]. Therefore, our objective was to determine the impacts of SOD1 and GPX1 knockouts on femoral activities of alkaline phosphatase and tartrate resistant acid phosphatase and mechanical properties by three-point bending in growing female mice.

2 Materials and methods

2.1 Mice

The GPX1(−/−) and SOD1(−/−) mice and their wild-type (WT) controls were originally developed by Dr. Y-S. Ho (Wayne State University, Detroit) and shared the same genetic background (129/SVJ × C57BL/6) [32]. All experimental mice (female, 9-wk old, $n = 7–8$ *per* genotype) used in this study were bred in our mouse facility and fed a Se-adequate (0.4 mg Se/kg) torula yeast diet [33]. Mice were given free access to food and distilled water, and housed in shoebox cages in a constant temperature (22°C) animal room with a 12-h light/dark cycle. All experiments were approved by the Institutional Animal Care and Use Committee at Cornell University and conducted in accordance with National Institute of Health guidelines for animal care.

2.2 Sample collection and preparation

Mice were euthanized by carbon dioxide asphyxiation followed by exsanguination with a heparinized syringe. Body mass of live animals was recorded immediately prior to euthanasia. Femora were removed. Left femora were collected for enzyme activity assays. In preparation, femora samples were cleaned, rinsed and extracted in 0.1 g/L triton-X-100 at 4°C for 72 h. The bone extractions were then vortexed, centrifuged, and the supernatants were stored at −80°C before assays. Right femora were stored at −20°C for mechanical testing.

2.3 Enzyme activity assays

Alkaline phosphatase and tartrate resistant acid phosphatase activities were measured based on the cleavage of *p*-nitrophenyl phosphate at pH = 10.3 and 5.5, respectively [34], and the colored reaction was measured at 405 nm. Results are expressed as the mean micromoles of *p*-nitrophenyl phosphate hydrolyzed *per* hour *per* milligram of cell protein. GPX1 activity was measured by the coupled assay of NADPH oxidation using hydrogen peroxide as a substrate as previously described [33]. The enzyme unit of GPX1 is defined as 1 nmol of reduced glutathione oxidized. The total SOD activity was measured using a water-soluble formazan dye kit (Dojindo Molecular Technologies). Mn-SOD activity was determined by the same method after treating the samples with 4 mM KCN for 30 min. SOD1 activity was determined by subtracting the Mn-SOD activity from the total SOD activities. Protein level was determined as described by Lowry *et al*. [35].

2.4 Mechanical testing

Whole bone structural performance was measured in three-point bending to failure. Each right femur was microradiographed prior to testing to record the bone geometry. Bone length and the major and minor, outer and inner diameters were measured from the radiograph. The cross-sectional moment of inertia (I, mm^4) was calculated assuming a hollow elliptical cross-sectional geometry. A 7-mm span was used for all three-point bending tests. The posterior surface of the femur was placed on the load supports and the load was applied anteriorly at the mid-diaphysis at 0.05 mm/s to failure. For this configuration, the anterior surface is in compression and the posterior aspect experiences tension. Load and displacement were collected at 10 Hz throughout the test. The failure bending moment (M_f, N/mm), displacement to failure (D_f, mm), and bending stiffness (EI, N/mm^2) were calculated from the load-displacement data. The effective modulus was calculated by normalizing the bending stiffness by the moment of inertia. To account for differences in body size [36], the failure moment was normalized by body mass (M_f/BM, N/mm/g).

2.5 Statistical analysis

All data were analyzed by one-way ANOVA for the effect of genotype followed by a Fisher's PLSD *post hoc* analysis

Table 1. Effects of GPX1 or SOD1 knockout on femoral bone enzyme activities[a)]

	WT	GPX1(−/−)	SOD1(−/−)
Glutathione peroxidase 1, nmol glutathione oxidized *per* min *per* mg protein	133.4 ± 7.1	16.1 ± 6.2*	68.7 ± 7.3*
Superoxide dismutase 1, 50% formazan dye formation rate inhibition *per* mg protein	210.8 ± 14.5	211.5 ± 10.3	2.6 ± 0.7*
Mn-Superoxide dismutase, 50% formazan dye formation rate inhibition *per* mg protein	3.4 ± 0.5	3.4 ± 0.4	2.5 ± 0.4
Alkaline phosphatase, μmol of *p*-nitrophenol released *per* min *per* mg protein	328.8 ± 68.1	468.0 ± 57.2*	410.4 ± 58.3
Tartrate resistant acid phosphatase, μmol of *p*-nitrophenol released *per* min *per* mg protein	6.8 ± 1.6	10.9 ± 1.4*	9.9 ± 1.3*

a) Values are means ± SE of 7–8 *per* genotype.
* $p < 0.05$ *vs.* WT.

Table 2. Effects of GPX1 or SOD1 knockout on femoral bone mechanical properties[a)]

	WT	GPX1(−/−)	SOD1(−/−)
Body mass (g)	19.9 ± 2.6	20.0 ± 0.8	15.1 ± 1.5*
Bone length (mm)	14.5 ± 0.3	14.5 ± 0.1	13.4 ± 0.3*
Failure bending moment (N/mm)	12.6 ± 2.2	13.9 ± 5.3	7.4 ± 1.2*
Bending stiffness (N/mm^2)	524.7 ± 87.1	421.4 ± 61.3	327.3 ± 21.7*
Cross-sectional moment of inertia (mm^4)	0.21 ± 0.05	0.22 ± 0.04	0.11 ± 0.02*
Displacement to failure (mm)	1.42 ± 0.34	0.99 ± 0.33	1.75 ± 0.41
Failure bending moment/body mass (N/mm/g)	0.61 ± 0.04	0.70 ± 0.15	0.49 ± 0.06*
Bending stiffness/cross-sectional moment of inertia$^{3/4}$ (N/mm^2)	3021 ± 895	2015 ± 334	3613 ± 612

a) Values are means ± SE of 7–8 *per* genotype.
* $p < 0.05$ *vs.* WT.

with significance level set at $p < 0.05$ (Statview, SAS Institute).

3 Results

3.1 Enzymes activities

Compared to the WT mice, femoral SOD1 activity in SOD1(−/−) mice and GPX1 activity in GPX1(−/−) mice was reduced ($p < 0.001$) by 98 and 88%, respectively (Table 1). While femoral GPX1 activity in SOD1(−/−) mice was 49% lower ($p < 0.05$) than that in the WT mice, femoral SOD1 activities were similar between the GPX1(−/−) and WT mice, and femoral Mn-SOD activities were not altered by genotype. SOD1(−/−) mice had 47% higher ($p < 0.05$) femoral tartrate resistant acid phosphatase activity than WT mice. However, the 25% increase in femoral alkaline phosphatase activity in SOD1(−/−) over the WT mice was only marginally significant ($p = 0.12$). In contrast, GPX1(−/−) mice had higher ($p < 0.05$) activities of both enzymes in femur than the WT mice.

3.2 Mechanical properties

Compared with the WT mice, SOD1(−/−) mice showed a 24% reduction ($p < 0.01$) in body mass and 8% ($p < 0.05$) in femoral length (Table 2). The cross-sectional moment of inertia about the plane of bending (I) and the failure bending moment of femora in the SOD1(−/−) mice were 47 and 43% lower ($p < 0.05$) than that of the WT, respectively. After normalizing by body mass to account for size differences, SOD1(−/−) mice still had lower ($p < 0.05$) femoral failure bending moment than WT mice. Femoral bending stiffness was 32% lower ($p < 0.05$) in SOD1(−/−) than in WT mice. Femoral displacement to failure and effective modulus (EI/I) was not significantly different among genotypes. The GPX1 knockout had no significant effect on body mass or any of the femoral bending measures.

4 Discussion

Our most significant finding was that the knockout of SOD1 impaired femoral bending strength and stiffness in

growing female mice. In contrast, knockout of GPX1 did not result in significant changes of these properties relative to WT mice. Thus, our data indicate that SOD1 plays a more potent role in skeletal development and load-bearing function than GPX1 before skeletal maturity and peak bone mass are achieved [37]. Although the limited amount of biochemical analysis in the present study could not fully explain the genotype differences, the relative activity changes in the two phosphatases might be a contributing factor. Compared with the WT mice, the SOD1(−/−) mice exhibited 47% greater femoral tartrate resistant acid phosphatase activity, but similar femoral alkaline phosphatase activity. Meanwhile, the GPX1(−/−) mice had greater activities of both enzymes than the WT mice. Likely, the SOD1(−/−) mice had more active osteoclastic resorption than osteoblastic formation, resulting in impaired cortical bone integrity. However, the GPX1(−/−) mice might have a simultaneous up-regulation of osteoclastic and osteoblastic activities or an overall increased bone remodeling activity.

Knockout of SOD1 and GPX1 presumably causes elevation of intracellular superoxide anion and H_2O_2, respectively, although primary bone cells isolated from both knockout mouse lines showed higher levels of ROS than those from the WT mice (Roy and Lei, unpublished). While normal levels of superoxide anions generated by NADPH oxidase in osteoclasts [2, 38] are required to activate osteoclastic resoprtion [1, 39–42], excessive levels of superoxide anions may overactivate osteoclastic activity [40]. Stimulation of osteoclastic bone resorption by superoxide anions has been demonstrated *in vitro*, and this stimulation was inhibited by the addition of exogenous SOD [12]. Our results provide *in vivo* evidence for the role of SOD1 in superoxide anion-mediated osteoclastic activity and resultant impact on bone mechanical properties in growing mice.

Alterations of intracellular H_2O_2 tones seem to affect bone metabolism in both ways. While H_2O_2 was generated during osteoblast proliferation [43] and stimulated osteoclastic resorption *in vitro* [9], osteoclast formation was abrogated in RAW cells overexpressing GPX1 [15]. Thus, elevated intracellular H_2O_2 tone in GPX1(−/−) mice augmented both bone formation and resorption, leading to increased bone turnover, but no net change in the overall bone mechanical properties. This finding may explain the observation that the GPX1(−/−) mice had altered femoral phosphatase activities, but similar mechanical characteristics to the WT mice at the test age. Because elevated bone turnover following menopause may contribute to reduced bone strength and fracture [44], it will be interesting to investigate if the effects of GPX1 may be more pronounced in older animals or upon induced oxidative stress. All together, the comparative impacts of SOD1 and GPX1 knockout on femoral mechanical indicate that regulation of intracellular superoxide anions may be more critical for bone development and integrity than that of intracellular H_2O_2 in growing female mice. In many *in vitro* and epidemiological studies, effects of superoxide anions and H_2O_2 in bone metabolism have not distinguished [1, 2, 7, 11].

It is noteworthy that femoral GPX1 activity in SOD1 (−/−) mice was 49% lower than that of the WT mice. This impaired GPX1 activity due to SOD1 null is similar to what we have observed in other tissues [45]. While the mechanical alterations in bones from SOD1(−/−) mice did not seem to be directly related to the reduced GPX1 activity in the femur, metabolic changes in soft tissues associated with SOD1 deletion could affect skeletal development and function. In fact, alterations in neuromuscular functions have been reported in the SOD1(−/−) mice, particularly evident at 5–6 month of age [46]. However, in the present study we did not notice apparent behavioral and physiological motor deficits in the SOD1(−/−) mice. Thus, the observed changes in bone mechanical properties in these mice are most likely primary effects of SOD1 deletion.

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