Aging amplifies multiple phenotypic defects in mice with zinc transporter Zip14 (Slc39a14) deletion

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Abstract

Inflammation and zinc dyshomeostasis are two common hallmarks of aging. A major zinc transporter ZIP14 (slc39a14) is upregulated by proinflammatory stimuli, e.g. interleukin-6. We have evaluated the influence of age on the Zip14 KO phenotype using wild-type (WT) and Zip14 knockout (KO) mice. Aging produced a major increase in serum IL-6 concentrations that was dramatically augmented in the Zip14 KO mice. In keeping with enhanced serum IL-6 concentrations, aging produced tissue-specific increases in zinc concentration of skeletal muscle and white adipose tissue. Metabolic endotoxemia produced by Zip14 ablation is maintained in aged KO mice. Muscle non-heme iron (NHI) was increased in aged WT mice but not in aged Zip14 KO mice demonstrating NHI uptake by muscle is ZIP14-dependent and increases with age. NF-κB and STAT3 activation was greater in aged mice, but was tissue specific and inversely related to tissue zinc. Micro-CT analysis revealed that Zip14 KO mice had markedly reduced trabecular bone that was greatly amplified with aging. These results demonstrate that the inflammation-responsive zinc transporter ZIP14 has phenotypic effects that are amplified with aging.

Keywords

Inflammation; Sarcopenia; Signaling pathways; Interleukin-6; bone; growth
1. Introduction

Many mediators of the aging process are associated with inflammation and oxidative stress (Vasto et al., 2007; Frazzini et al., 2006). These consequences may be augmented if there is a concurrent low intake of zinc (Marcellini et al., 2006; Pepersack et al., 2001; Tudor et al., 2005). Proinflammatory conditions have been shown to influence zinc metabolism through cellular transport and intracellular zinc processing (Cousins et al., 2006). Many of the same mediators may influence aging. For example, it has been proposed that the proinflammatory cytokine IL-6 is a key factor in human age-associated diseases (Bennermo et al., 2004; Olivieri et al., 2006). A single nucleotide polymorphism (SNP) in the IL-6 gene is associated with an increased inflammatory response (Bennermo et al., 2004; Mocchegiani et al., 2008). IL-6 has been shown to regulate components of the homeostatic mechanisms for zinc. Specifically, IL-6 has been shown to influence metallothionein expression and cellular zinc accumulation (Schroeder and Cousins, 1990).

We have established that the zinc transporter ZIP14 (slc39a14), a zinc importer usually located at the cell surface, is regulated by proinflammatory cytokines, particularly IL-6 (Liuzzi et al., 2005). The regulation of Zip14 by IL-6 suggests ZIP14 expression could be influenced by aging. Mice with a knockout (KO) of Zip14 have a phenotype that includes metabolic endotoxemia and altered glucose regulation (Aydemir et al., 2012; Guthrie et al., 2015; Troche et al., 2016). Such phenotypes are associated with aging. Here we present the first evidence on the influence of aging in mice with an ablation of Zip14. The aged mice were 22-24 months old thus placing them in an age comparable to humans between 56 and 69 years of age (Flurkey et al., 2007). The data shows that aging increases ZIP14 expression in muscle, that proinflammatory signaling pathways are influenced by aging and ZIP14 expression and that aging drastically reduced femoral trabecular bone with Zip14 ablation.

2. Materials and Methods

2.1 Mice

Zip14 heterozygous mice of a C57BL/6J and 129SF1 mixed background were used as a breeding colony at the University of Florida (Aydemir et al., 2012). Both WT (Zip14^+/+) and KO (Zip14^-/-) male mice were used for experiments. Young mice were 8 to 16 weeks of age and aged mice were 22-24 months old. Based on experience with the Zip14 KO strain in terms of their proinflammatory status and their lower than normal body size, we decided to study the mice at 24 months of age. Mice were provided with commercial chow diet (Harlan 7912) and tap water ad libitum. Mice were anesthetized using isoflurane (Baxter, Deerfield, IL, USA) and euthanasia was via cardiac puncture and exsanguination. All murine protocols were approved by the University of Florida Institutional Animal Care and Use Committee.

2.2 Serum and Tissue analyses

Blood was collected in clot activator tubes (Capiject, Terumo Medical, Somerset, NJ, USA). Serum was obtained by centrifugation and stored at −80°C. Harvested tissues were snap-frozen in liquid nitrogen and stored at −80°C. Zinc concentrations were measured by flame atomic absorption spectrometry (AAS). Serum was diluted 1:5 in Milli-Q® water for AAS.
Tissues were weighed and digested in HNO$_3$ (90°C for 3 hours) and diluted (1:1 to 1:5) with Milli-Q® water for AAS (Troche et al., 2016).

Tissue aliquots were homogenized in TRIzol reagent (Life Technologies, Thermo Fisher Scientific, Waltham, MA, USA) with a Bullet blender (Next Advance, Averill Park, NY, USA) using either zirconium oxide or stainless steel beads. Quantitative real-time PCR (qPCR) was performed using Express One-Step Superscriptmix (Invitrogen, Carlsbad, CA, USA) and a StepOnePlus Real-Time PCR detection system (Applied Biosystems, Foster City, CA, USA). TBP mRNA and GAPDH mRNA (muscle) were used to normalize qPCR data (Aydemir et al., 2012).

Tissue aliquots were homogenized as above in RIPA or non-denaturing lysis buffer (Santa Cruz Biotechnology, Dallas, TX, USA) containing a protease/phosphatase inhibitor mix (Thermo Fisher Scientific, Waltham, MA, USA) and phenylmethanesulfonyl fluoride (Sigma Aldrich, St. Louis, MO, USA). Proteins were separated using 10% SDS-PAGE and transferred to nitrocellulose with transfer verification through Ponceau Red staining. Protein abundance was visualized by chemiluminescence (Super Signal West Pico, Thermo Fisher, Waltham, MA, USA) and digital imaging (Protein Simple, San Jose, CA, USA). Tubulin was used as the gel loading control. ZIP14 and ZIP8 polyclonal antibodies were produced in-house as previously described (Ryu et al., 2008; Liuzzi et al., 2005). NF-κB, phosphorylated NF-κB, STAT3, phosphorylated STAT3, ERK1,2, and phosphorylated ERK1,2 antibodies were purchased from Cell Signaling Technology (Boston, MA, USA). Tissue non-heme iron was measured using tissue homogenates by the ferrozine method (Rebouche, et al., 2004).

2.3 Evaluation of bone structure with micro-CT

Femurs from the left leg were removed and separated from adherent tissue and was stored at −80°C. Thawed bones were individually scanned using a Scano μCT scanner (μCT-40; Scano Medical AG, Bassersdorf, Switzerland) as described in detail by Cao and Picklo (2013). Cortical and trabecular bone of the mid-femur was analyzed.

2.4 Statistical Analysis

Reported data are as means ± SE. Comparison of WT vs. KO genotype was by Student’s t-test. Genotype and age designation were treated as independent effects. ANOVA analyses were performed using the PROC MIXED procedures of SAS version 9.2 (SAS Institute, Cary, NC, USA) and Prism InStat 3. Multiple treatment comparisons were analyzed using the Turkey-Kramer means separation, and significance was established at P ≤0.05.

3. Results

3.1 Zinc concentrations of specific tissues are altered in aged mice

Zinc concentrations in spleens of WT mice were significantly decreased with age (p<0.01). In contrast, zinc concentrations in WAT and skeletal muscle were significantly increased (p<0.05) in aged mice (Table 1). Brain zinc concentrations were also greater (p<0.03) in the aged mice. The concentrations in other tissues including bone showed no significant
differences, indicating tissue specificity in zinc accumulation with aging. Because of the potential for inflammation to influence spleen and muscle, we focused on these tissues using liver as the control.

3.2 Aging influenced serum zinc, endotoxin, IL-6 concentrations and spleen IL-6 mRNA

The Zip14 ablation reduced growth in both young and aged mice compared to WT mice as shown by body length (Supporting Fig. 1) and body weight (Fig. 1A). Of note is that Zip14 KO mice maintained body weight in contrast to WT mice. Muscle to body weight ratios of young male mice were less in the KO genotype, but were not measured in the aged mice. Serum endotoxin was significantly greater in the Zip14 KO mice. This signature of metabolic endotoxemia was maintained in the aged KO mice (Fig. 1B). Measurement of serum IL-6 at steady state, using a high sensitivity ELISA, revealed that the WT aged mice had greater levels than young WT mice. Aging greatly amplified the differential in serum IL-6 in Zip14 KO mice (Fig. 1C). The exact cellular source(s) of the elevated serum IL-6 is not known. It is of note that IL-6 mRNA levels in the spleen of the aged KO mice are double those of the other groups indicating splenic macrophages could be one site of origin of the elevated IL-6 (Fig. 1D).

3.3 Activation of pro-inflammatory signaling pathways

Activation of NF-κB, STAT3 and ERK1/2 was examined in spleen, muscle and liver of young and aged WT and KO mice. Aging increased p-NF-κB in all tissues (Fig. 1E). This effect was markedly less in muscle and liver of KO mice while it was amplified in spleen which may lead to higher IL-6 expression. Aging also increased p-STAT3 in spleen and muscle, but activation was not influenced by genotype. p-ERK1/2 was less in liver of aged mice (Fig. 1E), but was not changed in the other tissues. Insulin receptor phosphorylation was less in aged mice.

3.4 Aging and ZIP14 expression influences tissue zinc and non-heme iron concentrations

Our initial hypothesis was that since serum IL-6 levels increase with age, expression of Zip14, which is induced by IL-6 (Liuzzi et al., 2005), may increase with age enhancing zinc transport and retention in specific tissues. A comparison of steady state serum zinc concentrations revealed that these were significantly greater (p<0.02) in the Zip14 KO mice (Fig. 2A). There was no effect due to age. A comparison of tissue zinc levels in WT versus KO mice revealed significant (p<0.01) increases in muscle of aged WT mice (Fig. 2B and C). These levels were increased further in muscle of aged Zip14 KO mice (Fig. 2C). This suggests that zinc transport kinetics are differentially altered in a tissue-specific fashion with aging. NHI was not influenced by aging in liver. In contrast, however, NHI levels in muscle were influenced by aging in the Zip14 KO mice used for these experiments (Fig. 2D).

3.5 ZIP8 and ZIP14 are differentially expressed with aging

We examined ZIP14 and its closest homologue ZIP8 (Schmitt-Ulms et al., 2009) in tissues of young and aged WT and KO mice. Protein levels for ZIP14 in liver of WT mice were comparable (Fig. 3A). ZIP14 in muscle was much more abundant in aged mice (Fig. 3A). In contrast, muscle ZIP8 expression was greatly increased with aging (Fig. 3B). Liver ZIP8
levels were much greater in the Zip14 KO mice. Aging increased ZIP8 levels in WT and KO mice with aging. These data suggest multiple factors are involved regulation of these transporters with aging. Of note is that MT1 mRNA levels were not influenced in muscle or spleen by aging or genotype (Fig. 3C).

3.6 Growth, cortical bone volume/surface area, and trabecular bone are altered with aging in Zip14 KO mice

Bone morphology was influenced by both age and Zip14 expression. Specifically, cortical bone volume decreased in aged KO mice and bone surface area increased in aged KO mice (Fig. 4A and 4B). Both of these measurements indicated that Zip14 ablation reduced bone mass. The striking loss of trabecular bone of the femur in mice with the null Zip14 mutation is shown in the μCT 3D representations (Fig. 5A). While not pronounced in young Zip14 KO mice, the aged KO mice showed significant reductions in trabecular bone based on numerical indices of bone mineral density (Fig. 5B). In keeping with these results Ear Mesenchymal Stem Cells (EMSC) from Zip14 KO mice and cultured in osteogenic medium were less mineralized than WT counterparts (Supporting Fig. 2). The effect of LPS while dramatic in WT, was not significant in KO EMSC. Further evaluation of these cells through western blots showed that pSmad1/5, a key transducer of the Bone morphogenetic protein (BMP) pathway, was downregulated with KO.

4. Discussion

There is currently great interest in the relationship of zinc transporter activity and disease. The range is extensive and has implicated many of the 24 ZnT/ZIP proteins that transport zinc. It has been proposed that the large number of zinc transporters is necessitated because of the plethora of intracellular functions in which this essential micronutrient participates (Lichten and Cousins, 2009a). Examples for the ZIP transporters/disease relationship include adipose inflammation and ZIP14 (Troche et al., 2016), ZIP8 and ZIP14 and response to sepsis (Knoell et al., 2009; Wessels and Cousins, 2015), ZIP13 and Ehlers-Danlos syndrome (Jeong et al., 2012), ZIP14 and metabolic endotoxemia (Guthrie et al., 2015), ZIP8 and osteoarthritis (Kim et al., 2014) and mutant Zip14 and parkinsonism/dystonia (Tuschi et al., 2016). One report that relates ZIP transporter function to aging was an evaluation of Zip6 mRNA expression in immune cells (Wong et al., 2013). The findings reported here are the first to specifically target the phenotypes of a ZIP transporter that are accentuated with aging.

Among the tissues most frequently associated with disorders of human aging are skeletal muscle and bone/ connective tissues. In experiments reported here we compared the response of muscle to other tissues with respect to zinc homeostasis, proinflammatory signaling pathways and bone development. The animals in this study were at steady state and only males were compared. The aged mice used were 22-24 months old and are comparable to humans between 56 and 69 years of age (Flurkey et al., 2007). This age range is appropriate because potential therapeutic intervention studies to combat age-related disease routinely use mice that are about 22 months old to test effectiveness (Camporez et al., 2016). Of the original cohort of WT and KO mice reserved for this study, no mortality
was observed during the 22 month comparison period. Gross examination would however classify the KO mice as low-functioning compared to the WT mice.

The Zip14 KO genotype clearly handles aspects of aging differently than WT mice. We interpret the increases of serum zinc levels in both young and aged Zip14 KO mice as indicative of a decrease in ZIP14-mediated uptake of systemic zinc. Tissue zinc levels in muscle are greater in aged mice, however. The finding that increased circulating IL-6, a signature of aging (Oliveri et al., 2006), is substantially increased in Zip14 KO mice suggests that the loss of ZIP14 leads to overexpression of IL-6 production, most likely through loss of a feedback loop. As mentioned earlier, the spleen could be a source of circulating IL-6 as IL-6 mRNA is increased. Previously we have shown that ZIP14 regulates inflammatory signaling in adipose tissue (Troche et al., 2016). Adipose is a major source of the increased IL-6 associated with aging (Starr et al., 2009). Hence in the Zip14 KO mice, adipose tissue may be a contributor to increased systemic IL-6 observed with aging in our experiments here as this genotype has increased body fat (Aydemir et al., 2012).

Elevated muscle zinc concentrations are in agreement with the increased ZIP14 in muscle of WT mice. Greater levels of zinc in muscle of Zip14 KO mice suggest a compensatory zinc transport function has occurred. The upregulation of muscle ZIP8 could contribute to this increase. Aging clearly increased ZIP8 expression in muscle and also liver. Most research on ZIP8 expression/function has focused on immune cells, starting with its identification via inducibility by lipopolysaccharide (Begum et al., 2002). We identified that activation of human primary T-cells dramatically increased ZIP8 and influenced intracellular zinc and inhibition of calcineurin phosphatase activity (Aydemir et al., 2009). Migration of ZIP8 as distinct bands of 75KDa and 150KDa in western analysis were noted at that time. Multiple molecular weights were noted here for splenic, muscle and liver ZIP8 (Fig 3D). The difference in molecular weight of ZIP8 could be related to shedding of the ectodomains (Ehsani et al, 2011) or formation of a homo- or hetero-dimer. To our knowledge, the current experiments are the first to report ZIP8 expression in skeletal muscle and the first to show up-regulation with aging.

Muscle was selected as a focus for these studies because of the influence of aging and sarcopenia and our previous profiling of Zip transporter mRNAs that respond to proinflammatory stimuli. Following an LPS injection (18 hr) Zip14 mRNA was increased 18-fold in skeletal muscle (Aydemir et al., 2012) and Zip8 mRNA with a 4-fold increase was the only other Zip mRNA showing a major response to LPS treatment (unpublished data). This is of interest since ZIP14 and ZIP8 have the closest homology among the ZIP transporter family and comprise one branch of the LIV-1 subfamily (Schmitt-Ulms et al., 2009). ZIP14 has been localized to the plasma membrane and endosomes (Liuzzi et al., 2005; Guthrie et al., 2015; Troche et al., 2016) and ZIP8 has been detected at the plasma membrane, mitochondria and lysosomes (He et al., 2006; Besecker et al., 2008; Aydemir et al., 2009). Under in vitro conditions these ZIP transporters may facilitate the movement of zinc and in some cases iron, manganese and cadmium (Lichten and Cousins, 2009). The magnitude of these metal fluxes is likely dependent upon the available pool of free metal ion to serve as a substrate for transport.
Our new findings merge with a concept where NHI accumulates in muscle and may contribute to muscle decline-of-function and atrophy (Xu et al., 2010). We demonstrate here that NHI does not accumulate in muscle of male Zip14 KO mice as it does in male WT mice (Fig. 2D). This suggests ZIP14 is responsible for the NHI accumulation. Aging male mice show augmented ZIP14 and ZIP8 in muscle as shown in this report (Fig 3 A, B). ZIP8 can also transport iron in vitro, albeit to a more limited extent than zinc (Wang et al., 2012). The prevention of muscle NHI accumulation with Zip14 ablation suggests ZIP8 is not a factor in muscle NHI accumulation. In contrast, previously we observed that ablation of Zip14 increased NHI in liver of young Zip14 KO female mice (Aydemir et al., 2012). Collectively these findings suggest that accumulation of NHI in muscle and liver in mice is influenced by both gender and age. The data support the concept that gender differences in iron metabolism occur in murine models.

Recent studies have clearly established a link between murine osteoarthritis, zinc and ZIP8 (Kim et al., 2014). In this scenario, zinc increases MTF-1 activation and thereby upregulated expression of specific metalloproteases involved in bone matrix degradation. Most likely a comparable situation is not involved in the current experiments as Mt-1 mRNA expression did not differ as a function of age in mice of the Zip14 KO genotype. Mt-1 mRNA is a sentinel for MTF-1 activation. We have focused here on muscle and spleen. Aging may produce changes in labile zinc pools in specific cells, e.g. leukocytes, Approaches to address such issues directly are beyond the scope of this report.

The influence of aging on femoral bone density in the aged mice observed in our studies by several indices using micro-CT is in line with age-related bone loss in humans (Khosla, 2013). The mice in this study were males eliminated from our breeding colony and allowed to age. At 22-24 months of age they are comparable to humans aged 56-69 years (Flurkey et al., 2007). Of major emphasis is the marked reduction in trabecular bone in only the aged KO mice. The mechanism responsible is not currently established. ZIP14 could influence bone accretion or resorption through zinc regulated signaling systems. This could include a role for IL-6. Our preliminary finding with osteogenic differentiation of EMSC from WT and Zip14 KO mice would support that concept (Supporting Fig. 2). Upregulation of NF-κB activation in stem cells derived from the Zip14 KO mice supports that motion. We cannot rule out the involvement of another ZIP14-regulated process in the loss of trabecular bone in the aged Zip14 KO mice. It has been postulated, based on in vitro transfection studies and data from zebrafish, that the major role for ZIP14 is to transport Mn (Tuschl et al., 2016). If that hypothesis is correct, ZIP14 dysfunction could reduce biliary Mn elimination, causing Mn retention in the brain leading to neurological disorders. Nutritional Mn deficiency has been shown to produce skeletal abnormalities in animals however excess Mn has not been shown to influence bone (Underwood, 1977). Hence Mn deficiency or toxicity is not a likely factor in the loss of trabecular bone observed in the aged Zip14 KO mice as described in our report.

5. Conclusion

In summary, the data presented in this report show that aging influences zinc metabolism particularly in muscle (Fig. 6). This suggests some influence of proinflammatory cytokines
and related signaling pathways. Activation of NF-κB and STAT3 pathways in muscle is most likely involved. Ablation of ZIP14 accentuates the elevated circulating levels of proinflammatory IL-6 and produces metabolic endotoxemia. Increases in ZIP8 expression suggest this transporter is also dysregulated in aging. ZIP14 ablation eliminates the NHI accumulation in muscle that occurs in aging. Proper functioning of ZIP14 in bone is essential during aging to maintain trabecular and cortical bone density. ZIP14 may influence diseases of aging including sarcopenia and osteoporosis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors are grateful to Dr. Jay Cao of the USDA Grand Forks Human Nutrition Research Center for performing the micro CT scans of the bone and the resulting analyses. We also thank Matthew P. Beke for help with the manuscript.

Funding

This project was supported by the National Institute of Diabetes and Digestive and Kidney Diseases (Grant No R01-DK094244) and Boston Family Endowment Funds of the University of Florida Foundation to RJC.

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Exp Gerontol. Author manuscript; available in PMC 2017 December 01.


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Exp Gerontol. Author manuscript; available in PMC 2017 December 01.
Highlights

- Zip14 deletion augments the elevated plasma IL-6 of aging
- Muscle zinc is increased with aging
- Zip14 deletion decreases trabecular bone in aged mice
- Muscle ZIP14 expression is greatly increased with aging
- Aging increases expression of ZIP8, a ZIP14 homologue, in muscle and liver
Fig. 1.
Influence of aging and Zip14 ablation using young and aged WT and Zip14 KO mice on (A) body weight, (B) serum endotoxin, (C) serum IL-6, (D) splenic IL-6 mRNA and (E) inflammatory pathways in spleen, muscle and liver. (A)-(D) values are mean ± SE, n=4 per group. P< values are shown.
Fig. 2.
Influence of aging and Zip14 ablation using young and aged WT and Zip14 KO mice on (A) serum zinc concentration, (B) splenic zinc concentration, (C) muscle and liver zinc concentrations, (D) muscle and liver non-heme iron (NHI) concentrations. Values are mean ± SE, n=4 per group. P< values are shown.
Fig. 3.
Influence of aging and Zip14 ablation using young and aged WT and Zip14 KO mice on (A) muscle and liver ZIP14 using western blots normalized to tubulin, (B) spleen, muscle and liver ZIP8 using western blots normalized to tubulin (C) MT1 mRNA in muscle and spleen. Values are mean ± SE, n=4 per group.
Fig. 4. Cortical bone microstructure of midshaft femur of young and aged WT and Zip14 KO mice measured using micro-CT. (A) Representative 3D reconstructions of femur cross sections. (B) Estimates of cortical bone volume/total volume (BV/TV), bone surface/bone volume (BS/BV) and cortical thickness (TbTh). Values are mean ± SE, n=4 per group.
Fig. 5.
Trabecular bone microstructure of distal femur of young and aged WT and Zip14 KO mice measured using micro-CT. (A) Representative 3D reconstructions of trabecular architecture of the femur. (B) Estimates of trabecular bone volume/total volume (BV/TV), connectivity density (ConnD), trabecular thickness mgHA/cm$^3$ (TbTh) and bone mineral density (BMD). Values are mean ± SE, n=4 per group.
Fig. 6.
Summary of changes associated with aging and those associated with ablation of Zip14. Increased circulating IL-6 is a signature of aging that is augmented with ZIP14 ablation. Aging increases muscle zinc and non-heme iron and decreases bone mineral density. These are influenced with ablation of ZIP14. Metabolic endotoxemia is a signature of ZIP14 ablation, but is not changed in aging.
Table 1
Zinc Concentrations of specific tissues of young (3-4 month) and aged (22 month) WT mice.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Age</th>
<th>μg Zn/mg tissue</th>
<th>P-Value</th>
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</thead>
<tbody>
<tr>
<td>Spleen</td>
<td>Young</td>
<td>23.89 ± 0.26</td>
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<tr>
<td></td>
<td>Aged</td>
<td>22.10 ± 0.47</td>
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<tr>
<td>Adipose</td>
<td>Young</td>
<td>2.11 ± 0.13</td>
<td>0.04</td>
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<tr>
<td></td>
<td>Aged</td>
<td>2.73 ± 0.16</td>
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<tr>
<td>Muscle</td>
<td>Young</td>
<td>8.31 ± 1.27</td>
<td>0.05</td>
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<tr>
<td></td>
<td>Aged</td>
<td>12.35 ± 1.27</td>
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<tr>
<td>Brain</td>
<td>Young</td>
<td>5.52 ± 0.11</td>
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<tr>
<td></td>
<td>Aged</td>
<td>5.97 ± 0.11</td>
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<tr>
<td>Liver</td>
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<td>34.80 ± 1.36</td>
<td>0.4</td>
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<tr>
<td></td>
<td>Aged</td>
<td>34.75 ± 1.13</td>
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<td>Heart</td>
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<td>13.95 ± 1.10</td>
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<td></td>
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<td>Lung</td>
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<td>17.18 ± 1.45</td>
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<td>Pancreas</td>
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<td>Aged</td>
<td>27.71 ± 2.28</td>
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<tr>
<td>Jejunum</td>
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<td>Cecum</td>
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<td>Colon</td>
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<tr>
<td></td>
<td>Aged</td>
<td>21.05 ± 1.09</td>
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Zinc was measured by AAS.
Results are means ± S.E.M. of samples from 4 mice per age group.