PERSPECTIVES

Aging and Oxidative Stress: A New Look at Old Bone

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Abstract

Advancing age is a major contributor to a decline of bone mass and strength and, consequently, a rise in the incidence of bone fractures. Skeletal aging in a murine model closely recapitulates age-related skeletal changes in humans, including decreased osteoblast number as well as decreased bone mass and strength and is associated with increased oxidative stress. This Perspective reviews what is known about the effects of the aging process on bone and its cellular constituents. Specifically, it discusses how pathways activated by oxidative stress can contribute to the decrease in osteoblast number and bone formation that are the hallmarks of involutional osteoporosis. Activation of the p53/p66SHC signaling cascade in the skeleton by reactive oxygen species increases osteoblast/osteocyte apoptosis. On the other hand, reactive oxygen species stimulate the FoxO family of transcription factors that defend cells against oxidative stress. In line with this, loss of FoxO function in the skeleton increases oxidative stress and decreases bone mass. Conversely, gain of FoxO function specifically in osteoblasts decreases oxidative stress and increases bone mass. This Perspective also reviews the potential contribution of loss of sex steroids, glucocorticoids, as well as oxidized lipids to oxidative stress and skeletal involution.

Keywords: Aging; Oxidative stress; p66SHC; FoxO; Wnt signaling; Sex steroids; Lipid oxidation; Glucocorticoids

The Pathophysiology of Skeletal Aging in Humans and Mice

The primary manifestation of aging is an overall decline in the capacity of various organs, including the skeleton, to maintain homeostasis. Age is a critical risk factor for the development of cardiovascular and metabolic diseases like atherosclerosis, insulin resistance, and metabolic syndrome. Importantly, advancing age is also a major risk factor for a decline in bone mass and strength and, consequently, a rise in the incidence of bone fractures. Loss of cancellous bone mass in humans starts as early as the 3rd decade, well before any change in sex steroid production (1). Nonetheless, in women the loss of trabecular bone in the spine accelerates substantially after menopause, attesting to the adverse role of estrogen deficiency on skeletal homeostasis and its contribution to the acceleration of age-associated bone loss. Cortical bone loss begins to decline after the age of 50 in both sexes, albeit at a faster rate in women than in men, suggesting that this bone compartment is more sensitive to sex steroids. Recently, Zebaze and colleagues have shown that bone loss at peripheral sites in the first 15 years after menopause makes only a small contribution to total bone loss throughout life (2). The majority of cortical bone loss occurs after the age of 65 years, and is not driven solely by the fall in estrogen levels.

The key pathogenetic mechanism leading to age-related skeletal fragility is impaired bone formation. A gradual decrease in bone formation begins shortly after peak bone mass (3;4). Age-related bone loss is due primarily to an insufficient number of osteoblasts resulting from the exhaustion of multipotential mesenchymal stem cell progenitors (3;4) and the diversion of these
overexpression of thioredoxin antioxidants 
gonadectomy in mice is prevented by 
unpublished data) and increases osteoblast number 
formation rate. The loss of bone mass with age in 
trate. The loss of bone mass with age in 
C57BL/6 female or male mice is associated 
with increased oxidative stress in bone and 
diminished canonical Wnt signaling, a critical 
regulator of bone formation (5;9;10).

Conserved Pathways That Regulate Skeletal Aging: The Role of Reactive Oxygen Species

Progressive oxidative damage has long 
been considered as the fundamental 
mechanism of age-associated physiological 
function decline (11;12). Modest levels of 
cellular reactive oxygen species (ROS) – the 
radical forms of oxygen – are generated by 
the mitochondrial electron transport chain 
during normal metabolism. Progressive 
mitochondrial damage with age, however, 
may result in excessive ROS production that 
damages proteins, lipids, and DNA, leading 
to cell death (13;14). It is now known that 
generation of H$_2$O$_2$ is a controlled process, 
involved in complex intracellular signal 
transduction cascades that regulate cell 
proliferation, differentiation, and migration 
in response to growth factors (15). NADPH 
oxidase (Nox) is largely responsible for 
receptor-dependent H$_2$O$_2$ production. 
Activated Nox complexes assemble within 
discrete subcellular compartments and, 
along with localized inactivation of 
antioxidant enzymes like peroxiredoxin I, 
allow restricted H$_2$O$_2$ accumulation for cell 
signaling (16;17).

In support of the contention that oxidative stress plays an important role in the 
skeleton, we and others have demonstrated 
that the loss of bone caused by 
gonadectomy in mice is prevented by 
antioxidants (5;18;19). Moreover, 
overexpression of thioredoxin-1, a major 
intracellular antioxidant, prevented the increase in oxidative stress and 
attenuated the suppression of bone formation following 
 streptozotocin-induced diabetes in mice (20). Both osteoblast number and bone 
formation are decreased in mice treated with 
the pro-oxidant buthionine sulfoximine (19), 
and murine models of premature aging 
associated with oxidative damage exhibit 
osteoporosis (21;22). Importantly, the 
increased osteoblast apoptosis in the bone 
of aged mice is abrogated by administration 
of the antioxidant N-acetyl-cysteine (NAC) or 
catalase (10). Thus, oxidative stress 
appears to be a critical contributor to the 
adverse effects of aging on bone mass and 
strength, as is the case in other tissues (23).

The p53-p66$^{Shc}$ signaling pathway controls 
intracellular redox status and attenuates 
osteoblastic cell differentiation and survival

Mammalian cells respond to oxidative stress 
using either long-lasting protective 
responses or apoptosis as a clearance 
mechanism for damaged cells. Increased 
ROS and osteoblast apoptosis caused by 
aging in the murine skeleton are associated 
with an increase in the phosphorylation of 
p53 and p66$^{Shc}$ in bone (5). Conversely, 
apoptosis and p66$^{Shc}$ phosphorylation in 
aged mice decrease with antioxidant 
administration (10). p66$^{Shc}$, an isoform of 
Shc, plays an important role as a redox 
enzyme implicated in mitochondrial ROS 
generation and translation of oxidative 
signals into apoptosis (24). p66$^{Shc}$ localizes 
predominantly in the cytoplasm, with a small 
fraction (10-40%) in the mitochondria. 
Oxygen-derived free radicals activate the 
protein kinase C-$\beta$ isoform to induce Ser$^{36}$ 
phosphorylation of p66$^{Shc}$, promoting the 
translocation of the protein from the cytosol 
to mitochondria. In the mitochondria, p66$^{Shc}$ 
acts as a redox enzyme to increase the 
production of H$_2$O$_2$ and apoptosis (25). 
Importantly, global p66$^{Shc}$ deficiency 
decreases ROS production in murine bone 
(Almeida M, Han L, and Manolagas S, 
unpublished data) and increases osteoblast 
resistance to oxidative stress in a cell-
autonomous fashion (26).
In cell cultures, p53 activation by oxidative stress leads to an increase in p66<sup>Shc</sup> protein abundance, and phosphorylation of Ser36 of p66<sup>Shc</sup> is a prerequisite for oxidant stress-induced apoptosis (27). Either p53-null or p66<sup>Shc</sup>-null cells are relatively resistant to oxidant-induced apoptosis and exhibit lower endogenous ROS production and reduced oxidative damage to DNA as compared to wild-type cells. In line with this, mouse genetic studies have revealed that p53 negatively regulates osteoblastogenesis and bone formation (21;28;29). However, it remains unknown whether ROS are involved in the actions of p53 on bone.

One possible consequence of reduced ROS production is less damage to macromolecules, including DNA and protein, and thus a slower rate of aging, as well as protection against age-related diseases. In support of this theory, genetic deletion of p66<sup>Shc</sup> in the mouse leads to a 30% longer lifespan (30). Remarkably, p66<sup>Shc<sup>−/−</sup></sup> mice are more resistant to diabetes and exhibit a reduced risk of atherosclerosis and cardiovascular damage when submitted to a high-fat diet [reviewed in (24)]. p66<sup>Shc<sup>−/−</sup></sup> mice are also protected against acute tissue damage after hind limb ischemia. The possibility that p66<sup>Shc<sup>−/−</sup></sup> deletion also confers resistance to age-related bone loss is under current investigation by our group.

**FoxOs and the skeleton**

Activation of FoxOs and their transcriptional targets, in particular the peroxide-scavenging enzymes MnSOD and catalase, constitute part of cellular antioxidant defense mechanisms (31). Recent work by our group and others has established that FoxOs play an important role in skeletal homeostasis. Specifically, combined somatic deletion of FoxO1, 3, and 4 at 3 months, for just 5 weeks, resulted in increased oxidative stress in bone, and bone loss at both cancellous and cortical sites (32). The decreased bone mass was due to deficient bone formation and resulted from decreased osteoblast number and increased osteoblast apoptosis. In contrast, bone resorption was not affected. *Ex vivo* studies performed using cells from FoxO-deleted mice demonstrated that FoxOs have cell-autonomous effects on osteoblastic cells, including an increase in the basal rate of apoptosis of calvaria- and bone marrow-derived osteoblasts due to oxidative stress. Interestingly, bone marrow-derived stromal cells from FoxO-deleted mice also exhibited increased expression of PPARγ and, consequently, increased adipogenic capacity (32), as well as decreased number of colony forming units of osteoblasts (CFU-OB) (Ambrogini E, Almeida M, and Manolagas S, unpublished data). These results suggest that FoxOs might contribute to the cell lineage specification of early multipotent mesenchymal stem cells. Mice with a gain of function of FoxO3 in osteoblasts exhibited decreased oxidative stress and osteoblast apoptosis, as well as increased osteoblast number, bone formation rate, and bone mass. This evidence favors the idea that FoxOs promote osteoblast survival via antioxidant actions. In agreement with our findings, Rached et al. reported that deletion of FoxO1 in osteoblasts expressing col1a1 leads to a decrease in osteoblast number and bone mass as a consequence of increased oxidative stress (33). However, in contrast to our studies, deletion of FoxO1 decreased osteoblastic cell proliferation but did not affect apoptosis. FoxO1 deletion also decreased protein synthesis in osteoblasts, leading to diminished levels of glutathione and collagen. Nonetheless, the same mice exhibited a striking increase in the production of osteocalcin (34). The increase in osteocalcin along with a decrease in Esp, which encodes a thymosine phosphatase called OST-PTP, resulted in increased pancreatic β-cell proliferation, insulin secretion and insulin sensitivity, suggesting that FoxO1 may be a key modulator of the ability of the skeleton to function as an endocrine organ regulating glucose metabolism. Nonetheless, combined somatic deletion of FoxO1, 3, and 4 or combined deletion of FoxOs in osteoblast progenitor cells and their progeny has no effect on glucose homeostasis (Ambrogini E, Almeida M, and Manolagas S, unpublished data). Overall, these lines of evidence clearly show that FoxOs have critical functions in skeletal homeostasis (Fig. 1).
Fig. 1. The role of FoxOs in cells of the osteoblast lineage. Activation of FoxOs diverts β-catenin from TCF- to FoxO-mediated transcription. In osteoblast precursor cells, this mechanism leads to a decrease in Wnt-induced osteoblastogenesis. On the other hand, FoxOs prevent oxidative stress and, consequently, apoptosis of osteoblasts and osteocytes.

**Oxidative Stress and Wnt Signaling**

Studies from our group have also revealed that the expression of β-catenin/TCF target genes decreases and that of FoxO target genes increases with age in murine bone, along with an increase in markers of oxidative stress and decreased bone formation (9). In osteoblastic cell models, oxidative stress induces the association of FoxOs with β-catenin, and β-catenin is required for the stimulation of FoxO target genes. Notably, oxidative stress induced by H₂O₂ promotes FoxO-mediated transcription at the expense of Wnt/TCF-mediated transcription and osteoblast differentiation. The effect of H₂O₂ on TCF transcription is attenuated by increasing levels of β-catenin, strongly suggesting that a limited pool of active β-catenin is diverted from TCF to FoxO transcription under stress conditions. Studies in colon carcinoma cells confirmed that the same mechanism operates in these models (35). Taken together, these observations suggest that age-related bone loss might result, at least in part, from the suppressive effect of ROS on Wnt/β-catenin signaling via FoxO activation (Fig. 1). In line with this idea, mice with targeted expression of Wnt10b in the bone marrow had increased bone mass and no evidence of age-related loss of bone mass (36). Activation of FoxOs might also decrease β-catenin/TCF-mediated transcription via alternative mechanisms. Indeed, in neural stem cells FoxOs promote the transcription of the Wnt signaling inhibitors Sost, sFRP1, and sFRP2 (37). Oxidative stress might also decrease Wnt signaling via dephosphorylation of GSK-3β (38;39) and activation of Dkk1 transcription (40;41). Interestingly, however, Wnt signaling can also be activated by oxidative stress (42;43) and administration of antioxidants to cultured osteoblastic cells or mice decreases Wnt signaling and osteoblastogenesis, respectively (10).

While this evidence clearly indicates that oxidative stress modulates Wnt signaling, there is also data indicating that β-catenin promotes the expression of several antioxidant genes in different cell types and prevents oxidative stress in the liver (44-46). It remains unknown whether this function of β-catenin occurs in cells of the osteoblast lineage or whether it contributes to its osteoblastogenic properties.

**Systemic Hormones Like Sex Steroids and Glucocorticoids, as Well as Oxidized Lipids, Exert Their Influence on Skeletal Homeostasis, at Least in Part, via Regulation of Oxidative Stress**

*Age-associated oxidative stress is potentiated by loss of sex steroids*
Similar to aging, gonadectomy promotes an increase in ROS and the phosphorylation of p53 and $p66^{Shc}$ in bone (5;18;47). Estradiol, DHT, or several antioxidants completely reverse the effects of gonadectomy on these markers of oxidative stress. More strikingly, administration of antioxidants is as effective as sex steroid replacement in preventing the increased osteoclastogenesis, osteoblastogenesis, and increased osteoblast and osteocyte apoptosis, as well as the loss of BMD in gonadectomized female or male mice. In vitro studies in osteoblastic cells have elucidated that $p66^{Shc}$ is an essential mediator of the effects of oxidative stress, not only on apoptosis but also on NF-$\kappa$B activation and the expression of cytokines like TNF-$\alpha$ and IL6 (26). Sex steroids antagonize all these effects of oxidative stress by preventing PKC-$\beta$-induced $p66^{Shc}$ phosphorylation via a mechanism that requires ERKs (5) (Fig. 2). Importantly, binding of the estrogen receptor (ER) $\alpha$ to DNA is not required for the ability of this receptor to mediate the anti-oxidant effects of estrogens on bone (48).

Studies by several groups have shown that ROS are required for the formation and activation of osteoclasts [reviewed in (23)]. Consistent with the requirement of ROS in osteoclast generation and the in vivo evidence that sex steroids prevent bone loss via anti-oxidant effects, estradiol or DHT attenuates osteoclastogenesis and stimulates osteoclast apoptosis by a mechanism that involves activation of glutathione and thioredoxin reductases and inhibition of NF-$\kappa$B (5;18). Similar to the case with osteoblasts, the anti-osteoclastogenic and pro-apoptotic effects of estradiol on osteoclasts are independent of the DNA-binding function of the ER (49). Overall, these studies suggest that the loss of sex steroids contributes to age-related bone loss, at least in part by increasing oxidative stress.

Lipid oxidation amplifies oxidative stress and diminishes osteogenic Wnt signaling in the aged skeleton

Lipoxygenase-dependent formation of oxidized lipids plays an essential role in the development of atherosclerosis and epidemiologic evidence from humans as well as studies in mice point to a link
between osteoporosis and cardiovascular disease [reviewed in (23)]. The expression of the lipoxygenases Alox12, Alox12e, and Alox15, as well as the lipid oxidation product 4-HNE, and the adipogenic PPARγ, increases in the bone of C57BL/6 mice with advancing age (50). Moreover, lipoxygenases oxidize polyunsaturated fatty acids to form products that bind to and activate PPARγ. In the process, they generate pro-oxidants like 4-HNE (51). Both PPARγ and Alox15 influence skeletal homeostasis as demonstrated by evidence that BMD is increased in mice lacking Alox15 or in mice that are haploinsufficient for PPARγ (52;53). On the other hand, mice expressing high levels of Alox15 have low BMD (52), and activation of PPARγ with synthetic thiazolidinedione ligands decreases bone mass both in humans and in mice [reviewed in (54)].

The expression of Alox15 is stimulated by oxidative stress in cultured cells (55). In line with this, NAC or catalase completely reversed the age-related increase in Alox15 expression to levels found in younger mice (10). Importantly, other conditions leading to oxidative stress in the skeleton, like loss of estrogens (5;18) and deletion of FoxOs (32), also increase Alox15 expression in bone (Jilka R and Almeida M, unpublished data). Similar to aged mice, young mice expressing high levels of Alox15 exhibit increased oxidative stress, lipid peroxidation and PPARγ expression, as well as reduced Wnt signaling in the skeleton. 4-HNE activates FoxOs that in turn attenuate β-catenin/TCF-mediated transcription, as described above. Because TCF-mediated transcription suppresses PPARγ expression (56;57), inhibition of β-catenin/TCF transcriptional activity leads to an increase in PPARγ levels. Oxidized PUFAs promote PPARγ association with β-catenin and induce β-catenin degradation (50;58), thereby further decreasing β-catenin/TCF-mediated transcription (Fig. 3). Oxidized lipids also stimulate apoptosis of osteoblastic cells and inhibit BMP-2-induced osteoblast differentiation (59-61). Via these mechanisms, lipid oxidation contributes to the decline in osteoblast number and bone formation that occurs with aging.

Endogenous glucocorticoids contribute to the age-related loss of bone mass and strength

An increase in the production of endogenous glucocorticoids with age, as well as enhanced sensitivity of bone cells to glucocorticoids, represents another age-associated pathogenetic mechanism of involutional osteoporosis (62). Aging also decreased the volume of the bone vasculature and solute transport from the peripheral circulation to the lacunar-canicular system. Furthermore, mice with osteoblast/osteocyte-specific transgenic expression of 11β-HSD2, the enzyme that inactivates glucocorticoids, are protected from the adverse effects of aging on osteoblast and osteocyte apoptosis, bone formation rate and microarchitecture, crystallinity, vasculature volume, interstitial fluid, and strength. Moreover, exposure to glucocorticoids suppressed angiogenesis in fetal metatarsals, as well as hypoxia-inducible factor 1α transcription and VEGF production in osteoblast and osteocyte cultured cells. This, and the evidence that dehydration of bone decreases strength, strongly suggests that endogenous glucocorticoids increase skeletal fragility in old age as a result of cell-autonomous effects on osteoblasts and osteocytes, leading to a decrease in bone angiogenesis, vasculature volume, and osteocyte lacunar-canicular fluid.

Recently, we and others have found that glucocorticoids stimulate ROS production and FoxO activity (63-65). Indeed, suppression of oxidative stress reduces glucocorticoid-induced osteoblast apoptosis and FoxO activation in vitro. Moreover, in osteoblast progenitor cells, FoxOs mediate the inhibitory actions of glucocorticoids on Wnt signaling (Almeida M, Han L, and Manolagas S, unpublished data), suggesting that at least some of the deleterious actions of glucocorticoids on bone might be mediated by ROS (Fig. 3).

Outlook

Mitochondrial function and ROS have been consistently implicated in many diseases of
Fig. 3. Mechanisms that contribute to the development of involutional osteoporosis. Skeletal aging in mice is associated with increased oxidative stress, lipid oxidation, and sensitivity to endogenous glucocorticoids. Increased reactive oxygen species (ROS) induce osteoblast/osteocyte apoptosis via activation of p66Shc and also stimulate FoxO transcriptional activity. Activation of FoxOs decreases β-catenin/TCF-mediated transcription and osteoblastogenesis. Lipid oxidation via 4-HNE contributes to ROS generation. The expression of the lipoxygenase Alox15 is increased by ROS and feed forward on lipid oxidation. The oxidized lipids generated by this process bind to and activate PPARγ, further contributing to a decrease in β-catenin/TCF-induced osteoblastogenesis. Activation of PPARγ also leads to increased bone marrow adiposity. The increased glucocorticoid sensitivity of osteoblast/osteocytes with age leads to decreased bone angiogenesis, vasculature volume, and osteocyte lacunar-canalicular fluid, as well as decreased strength. Glucocorticoids also increase ROS levels in bone.

aging. However, conventional untargeted antioxidants have not demonstrated a clear benefit in human studies. It is now well-established that upregulation of the cellular antioxidant system protects cells from oxidative damage, but it also alters the redox signaling required for signal transduction initiated by many growth factors. Consistent with this, studies in C. elegans indicate that fine-tuning redox signaling to meet an optimal cellular response to certain ROS levels is of crucial importance to longevity (66). In mice, administration of NAC and/or catalase for 4 weeks did not increase bone mass or strength in aged mice, despite the ability of these agents to suppress oxidative stress and osteoblast apoptosis (10). Consistent with the evidence that the propagation of Wnt signaling is rapidly and transiently stimulated by ROS (42), antioxidants exerted negative effects on osteoblastogenesis in vivo, and inhibited Wnt signaling in vitro (10;18). This mechanism might override the positive effect of antioxidants on Wnt/β-catenin signaling resulting from abrogation of ROS-induced FoxO activation (9;50). Perhaps more importantly, antioxidants inhibit the generation and activity of osteoclasts, leading to a reduction in bone remodeling (18;67). Thus, while effective in preventing the loss of bone mass in situations of high remodeling like in estrogen deficiency, conventional antioxidants do not restore bone mass in aged mice. Nevertheless, an emerging class of small-molecule antioxidants targeted to the mitochondria, which can mimic the activity of endogenous antioxidant enzyme systems and
subsequent physiological effects on pathways mediated by ROS, hold great promise for future therapeutic use in age-related diseases (68). In the meantime, the evidence demonstrating that ROS can decrease β-catenin/TCF signaling via FoxO activation suggests that the deleterious actions of oxidative stress in the skeleton are determined by specific signaling functions of ROS. Future studies targeting the pathways affected by ROS in bone cells should elucidate the molecular mechanisms that are relevant to age-associated skeletal involution, and may also identify novel drug targets for the prevention/treatment of age-related bone loss.

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