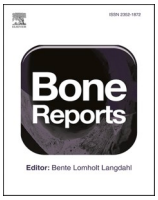
Bone Reports 19 (2023) 101664

Contents lists available at ScienceDirect

Bone Reports

journal homepage: www.elsevier.com/locate/bonr



The role of reactive oxygen species in bone cell physiology and pathophysiology

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ARTICLE INFO

Keywords:
Aging
Estrogen
FoxOs
Mitochondria
NOXs
Osteoblasts
Osteoclasts

ABSTRACT

Hydrogen peroxide (H_2O_2), superoxide anion radical ($O_2^{\bullet-}$), and other forms of reactive oxygen species (ROS) are produced by the vast majority of mammalian cells and can contribute both to cellular homeostasis and dysfunction. The NADPH oxidases (NOX) enzymes and the mitochondria electron transport chain (ETC) produce most of the cellular ROS. Multiple antioxidant systems prevent the accumulation of excessive amounts of ROS which cause damage to all cellular macromolecules. Many studies have examined the contribution of ROS to different bone cell types and to skeletal physiology and pathophysiology. Here, we discuss the role of H_2O_2 and $O_2^{\bullet-}$ and their major enzymatic sources in osteoclasts and osteoblasts, the fundamentally different ways via which these cell types utilize mitochondrial derived H_2O_2 for differentiation and function, and the molecular mechanisms that impact and are altered by ROS in these cells. Particular emphasis is placed on evidence obtained from mouse models describing the contribution of different sources of ROS or antioxidant enzymes to bone resorption and formation. Findings from studies using pharmacological or genetically modified mouse models indicate that an increase in H_2O_2 and perhaps other ROS contribute to the loss of bone mass with aging and estrogen deficiency, the two most important causes of osteoporosis and increased fracture risk in humans.

1. Introduction

Reactive oxygen species (ROS) refers to a large family of oxidants formed by the partial reduction of molecular oxygen (Lushchak, 2014; Sies and Jones, 2020). ROS is a collective term for a number of non-radical and free radical species (with at least one free electron) with very divergent reactivity. These include hydrogen peroxide (H_2O_2), organic hydroperoxides (ROOH), superoxide anion radical ($O_2^{\bullet-}$), and hydroxyl radical ($^{\bullet}OH$), among others. It is well established for more than 50 years that the major oxidant in redox regulation of biological activities is H_2O_2 (D'Autreaux and Toledano, 2007; Winterbourn, 2018). The H_2O_2 intracellular concentration is tightly controlled within the range of few nanomolar (Parvez et al., 2018). Various metabolic signals such as chemokines, growth factors, or physical stressors stimulate the production of H_2O_2, while its removal is accomplished by multiple reducing systems. Maintenance of low-levels of H_2O_2 and its physiological redox signaling has been recently coined as 'oxidative eustress' (Sarsour et al., 2014; Sies, 2017). For example in stem cells, physiological levels of H_2O_2 determine the balance between proliferation, self-renewal and differentiation (Tan and Suda, 2018). Higher levels of H_2O_2 favor increased exit from quiescence and increased proliferation with subsequent differentiation. Elevation of H_2O_2 concentration has also been associated with replicative senescence of stem cells. On the other hand, supraphysiological levels of H_2O_2 (roughly above 100 nM) causes unspecific oxidation of proteins and reversible and irreversible damage to lipids, proteins and nucleic acids, leading to inhibition of proliferation and cell death, a state referred to as 'oxidative distress' (Sies et al., 2017).

In this Review, we focus on the role of H_2O_2 and $O_2^{\bullet-}$ and their major enzymatic sources in the bone cells that directly contribute to bone remodeling, namely osteoclasts and osteoblasts. We will also address cellular and molecular mechanisms that impact and are altered by ROS. Finally, we discuss the contribution of ROS to the bone loss that occurs with aging and estrogen deficiency. Other cells in bone such as chondrocytes are greatly impacted by ROS however these will not be addressed here and the readers are directed to excellent reviews on this

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https://doi.org/10.1016/j.bonr.2023.101664
Received 20 January 2023; Received in revised form 17 February 2023; Accepted 20 February 2023
Available online 24 February 2023

topic (Bolduc et al., 2019; Wegner and Haudenschild, 2020).

2. ROS generation and elimination

The major producers of H_2O_2 and $O_2^{-\bullet}$ are the NADPH oxidases (NOX) enzymes and the mitochondria electron transport chain (ETC) (Schroder, 2020) (Fig. 1). NOXs are membrane-bound proteins that transfer electrons from nicotinamide adenine dinucleotide phosphate (NADPH) to molecular oxygen (Cross and Segal, 2004). Seven NOX enzymes have been described. NOX1, NOX2 (originally termed gp91phox), and NOX3 exist as a complex with $p22^{phox}$. Signals that activate these enzymes such as inflammatory mediators, cause the assembly of an active cytoplasmic complex containing Rac along with other activating proteins. NOX4 is constitutively active and also requires $p22^{phox}$ but not other regulatory subunits. NOX5 and the dual oxidases DUOX1 and DUOX2 are activated by Ca^{2+} through a cytoplasmic calcium-binding domain. The primary product of NOX1, NOX2, NOX3, and NOX5 is superoxide, while the primary product of NOX4, DUOX1, and DUOX2 is H_2O_2 (Lambeth and Neish, 2014).

In addition, the ETC produces $O_2^{-\bullet}$, which is then converted to H_2O_2 (Brand, 2020; Murphy, 2009). During the transport of electrons through the ETC, electrons can leak and react with molecular oxygen to generate $O_2^{-\bullet}$ (Cadenas and Davies, 2000; Turrens, 2003) (Fig. 1). Complex I and complex III are the major contributors of ROS (Brand, 2010). Complex I produces $O_2^{-\bullet}$ in the matrix while complex III produces $O_2^{-\bullet}$ towards the matrix and the intermembrane space (Kussmaul and Hirst, 2006; Murphy, 2009). $O_2^{-\bullet}$ is rapidly converted to H_2O_2 by Cu/Zn superoxide dismutase (SOD1) and manganese superoxide dismutase (SOD2). SOD1 is expressed mainly in the cytosol but also in the intermembrane space while SOD2 is expressed in the mitochondrial matrix (Fukai and Ushio-Fukai, 2011; Levanon et al., 1985; Wan et al., 1994). A recent study performed in a myoblastic cell line has estimated that about 40 % of net cellular H_2O_2 production is from NOXs and about 45 % from the ETC, and the remainder from other enzymatic sources (Wong et al., 2019).

H_2O_2 is further reduced to water by catalase, peroxidases, including glutathione peroxidase (GPx) and peroxiredoxins (Prdx) present in different subcellular compartments. Catalase is found primarily in peroxisomes (Kirkman et al., 1999). GPx converts H_2O_2 to H_2O, in a process that requires the oxidation of glutathione (GSH). The oxidized glutathione (GSSG) is reduced to GSH by the activity of glutathione reductase, a reaction that is coupled to the oxidation of NADPH to $NADP^+$ (Forman et al., 2009). The GSH/GSSG ratio is a good redox indicator and alterations in this ratio have been associated with oxidative distress in several pathological conditions (Alkazemi et al., 2021; Patel et al., 2016). Prxd1, 2, 4, and 5 are cytosolic whereas Prxd3 is present in the mitochondria. The activity of Prdx required the reduction of thioredoxins (Trxs), which also uses NAPDH as a co-factor.

3. Redox sensitive targets

H_2O_2 signals via reversible oxidation of cysteine residues in target proteins. This process, in turn, modulates phosphorylation events and critical cellular functions such as metabolism and replication (Holmstrom and Finkel, 2014; Parvez et al., 2018). A recent study has systematically identified sites of cysteine modification throughout the proteome of 10 distinct mouse tissues (Xiao et al., 2020). Various transcription factors seem to be redox sensitive. Some of the well-established redox hubs described in mammalian cells include nuclear factor erythroid 2 (NRF2), nuclear factor-κ light chain-enhancer of activated B cells (NF-κB), and forkhead box O transcription factors

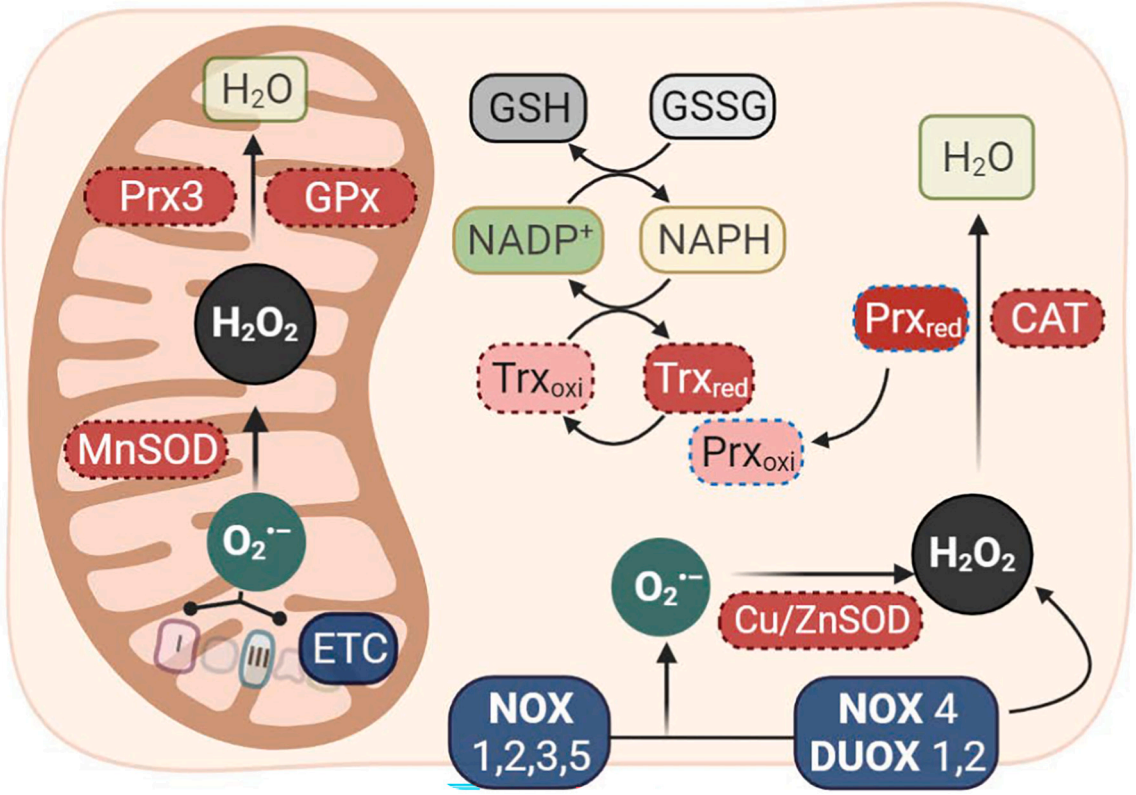

Fig. 1. Cellular sources reactive oxygen species and antioxidant defenses. The major sources of hydrogen peroxide (H_2O_2) and superoxide anion ($O_2^{-\bullet}$) are the electron transport chain (ETC) in the mitochondria and the NADP oxidases (NOXs) at the plasma membrane. Key antioxidant enzymes (red boxes) and co-factors are present in different cellular compartments and reduce H_2O2 to water. These systems are hither described in the text. Abbreviations: Cat: catalase; Cu/ZnSOD: copper-zinc superoxide dismutase; DUOX, dual oxidase; GSH: glutathione; GSH-Px: glutathione peroxidase; GSSG: glutathione disulfide; MnSOD: manganese superoxide dismutase; NADP: nicotinamide adenine dinucleotide phosphate. NADP+: oxidized (oxi); NAPH: reduced (red) form; Prx: peroxiredoxins; Trx: thioredoxin.

(FOXOs) (Klotz et al., 2015; Mitchell et al., 2016; Shi and Dansen, 2020; Yamamoto et al., 2018). As appropriate, we describe below the contribution of these different transcription factors to skeletal homeostasis.

Signaling through $O_2^{-\bullet}$ is less understood. One of the known roles for $O_2^{-\bullet}$ is the disruption of the Fe—S cluster in aconitase, a TCA cycle enzyme, which affects mitochondrial activity (Buettner, 2011; Lushchak et al., 2014). Signaling induced by oxidative distress can also be conducted far from its sources by conversion to more stable species, such as lipid peroxides or 4-hydroxynonenal (4-HNE) (Zhang and Forman, 2017). Indeed, phospholipids containing polyunsaturated fatty acids are susceptible to lipid peroxidation caused by ROS. Oxidized phospholipids are present on the surface of apoptotic cells and low-density lipoproteins (OxLDL) and their levels increase in many inflammatory states (Binder et al., 2016).

4. Oxidants in bone cell physiology

Genetic and pharmacological approaches have been used to modulate the subcellular levels of oxidants or to examine the function of a specific oxidant and assess its impact on physiology. The laboratory of Peter Rabinovitch has generated mice that express catalase targeted to different subcellular compartments including the nucleus (nCAT), mitochondria (mitoCAT), or peroxisomes (pCAT) to determine the effects of H_2O_2 particularly in the context of aging and age-related diseases (Schriner et al., 2005). MitoCAT mice, but not nCAT or pCAT mice, have an increase in median and maximal lifespan (Schriner et al., 2005) and attenuated insulin resistance, atherosclerosis, cardiac failure, pulmonary hypertension, muscle atrophy, and various types of cancers (Dai et al., 2017). Furthermore, mice with conditional deletion of SOD1, SOD2, or NOXs have been used to dissect the contribution of these different oxidant-generating enzymes in physiology. Some of these mouse models have been utilized to examine the role of oxidants in bone cells and bone mass. In addition to these genetic models, experiments with administration of antioxidants have evaluated the role of ROS in the context of sex-steroid deficiency and aging.

4.1. ROS in osteoclast lineage cells

Over twenty years ago, experiments with calvaria organ cultures have shown that osteoclast formation in response to parathyroid hormone and interleukin-1 is associated with superoxide anion generation. This work identified a role for ROS in osteoclast differentiation and bone resorption (Garrett et al., 1990). It was later found that both M-CSF and RANKL – the two indispensable cytokines for osteoclast formation – increase the levels of ROS which, in turn, potentiate osteoclast formation, activation, and survival (Bhatt et al., 2002; Ha et al., 2004; Lee et al., 2005) (Fig. 2). An increase in osteoclast ROS has been associated with mitochondria biogenesis orchestrated by peroxisome proliferator-activated receptor-gamma coactivator-1 beta (PGC-1β) in mice (Ishii et al., 2009). Conditional deletion of proteins involved in different aspects of mitochondria biology in osteoclast lineage cells in mice have highlighted the relevance of optimal mitochondria function to bone resorption (Ballard et al., 2020; Ishii et al., 2009; Jin et al., 2014; Miyazaki et al., 2012). Moreover, in osteoclast precursors RANKL rapidly increases complex I activity and mitochondrial respiration, well before the effects on PGC-1β expression and mitochondria content can be detected (Kim et al., 2020a). A role for mitochondrial H_2O_2 in osteoclast differentiation and activity was examined with a mouse model expressing the mitoCAT transgene in osteoclast lineage cells (Bartell et al., 2014). These animals have high bone mass due to a decrease in osteoclast numbers (Table 1). Accordingly, macrophages from mitoCAT mice exhibit low proliferative capacity and form less osteoclasts in culture. In addition, mature osteoclasts display increased apoptosis.

Cytoplasmic ROS is also thought to contribute of osteoclastogenesis. Numerous in vitro studies have suggested that ROS produced by NOX1, NOX2 and NOX4 participate in osteoclast differentiation (Lee et al., 2005; Schroder, 2019; Wegner and Haudenschild, 2020). RANKL decreases NOX2 and increases NOX1 and NOX4 expression (Sasaki et al., 2009; Sun et al., 2021). Mice deficient in NOX2 ($gp91^{phox}$) have a modest increase in bone mass and impaired osteoclast differentiation due to attenuation of $O_2^{-\bullet}$ and defective expression of NFATC1 (Kang and Kim, 2016). Goettsch and colleagues found that $NOX4^{-/-}$ mice have low osteoclast number and increased trabecular thickness (Goettsch et al.,

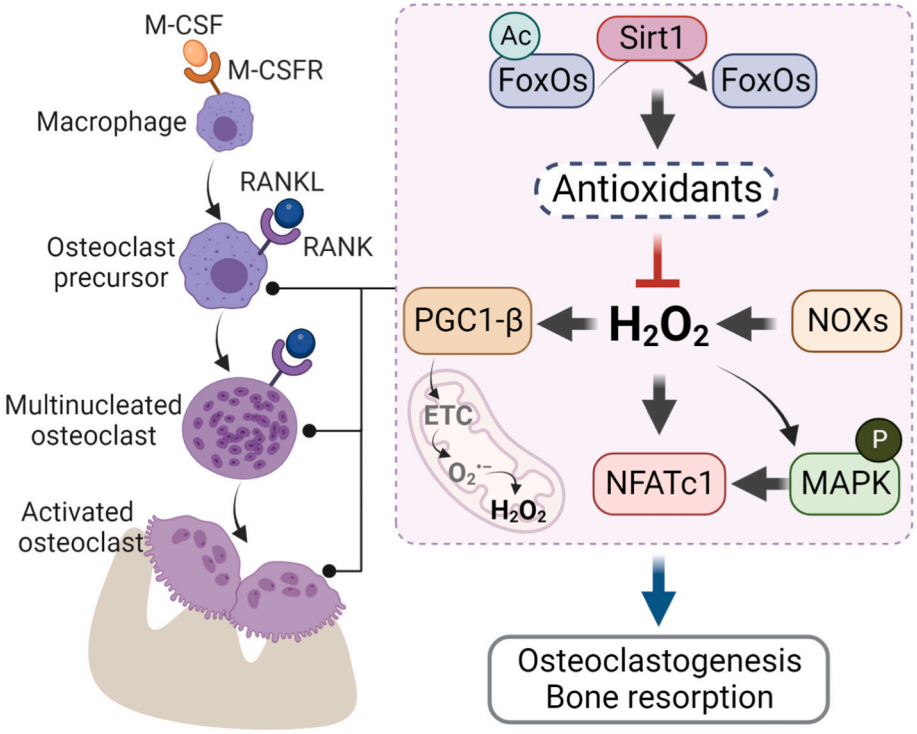

Fig. 2. Role of ROS in osteoclast lineage cells. Scheme showing osteoclast differentiation from hematopoietic precursors and the main cellular processes that interfere with hydrogen peroxide (H_2O_2) levels and affect osteoclastogenesis and bone resorption. Abbreviations: AcFoxO: acetylated forkhead homeobox type 0; ETC: electron transport chain; NFATc 1: nuclear factor of activated T-cells; NOX, NADP oxidase; O_2: superoxide; PGC1β: peroxisome proliferator-activated receptor-gamma coactivator-1 beta; P MAPK: phosphorylated mitogen-activated protein kinase; Sirtl: Sirtuinl.

Table 1
Mouse models of ROS effects on the skeleton.

Mouse model	ROS targeted	Bone cells affected	Bone mass	Pathophysiological bone loss	References
NAC or ascorbate administration to mice	↓ H_2O_2	↓ Osteoclasts and osteoblasts	Unknown	Attenuates bone loss with estrogen deficiency	Almeida et al., 2007 Lean et al., 2003
mitoCAT	↓ Mitochondrial H_2O_2	Unknown	↓	Not protected against bone loss with combined hind limb unloading and irradiation	Schreurs et al., 2020
mitoCAT; LysM-Cre	↓ Mitochondrial H_2O_2	↓ Osteoclasts	↑	Attenuates bone loss with estrogen deficiency Not protected against bone loss with aging	Bartell et al., 2014 Ucer et al., 2017
mitoCAT; Prx1-Cre	↓ Mitochondrial H_2O_2	Progenitor cells, stromal cells, osteoblasts, and osteocytes	NC	Attenuates bone loss with skeletal aging	Ucer et al., 2017
gp91$^{phox-/-}$	↓ $O2^{-\bullet}$	↓ Osteoclasts	↑	Not tested	Kang and Kim, 2016
Nox4$^{-/-}$	↓ H_2O_2	↓ Osteoclasts NC	↑ NC	Not protected against bone loss with estrogen deficiency	Goettsch et al., 2013 Watt et al., 2018
PrxCre$^{+/-}$ Nox4$^{fl/fl}$	↓ H_2O_2	Serum markers OC and OB ↑ NC ↓[a]	↑ NC ↓[a]	Not tested	Chen et al., 2022
Prdx2 KO	↑ H_2O_2	Unknown	↑	Not tested	K.M. Kim et al., 2019
Sod1$^{-/-}$	↑ Cytoplasmic $O_2^{-\bullet}$	↓ Osteoblasts and osteoclasts	↓	Exacerbates bone loss with mechanical unloading	Nojiri et al., 2011 Morikawa et al., 2013
Dmp1CreSod2$^{fl/fl}$	↑ Mitochondrial $O_2^{-\bullet}$	↓ Osteoblasts ↑ Osteoclasts	↓	Not tested	Kobayashi et al., 2015
Runx2CreSod2$^{fl/fl}$	↑ Mitochondrial $O_2^{-\bullet}$	↓ Osteoblasts ↑ Osteoclasts	↓	Not tested	Schoppa et al., 2022
Nrf2$^{-/-}$	↑ H_2O_2	↑ Osteoclasts	↑ NC ↓[b]	Exacerbates bone loss with irradiation	Kim et al., 2014 Rana et al., 2012 Park et al., 2014 Pellegrini et al., 2017
Dmp1CreERt2Nrf2$^{fl/fl}$ Col1a1CreNrf2$^{fl/fl}$	↑ H_2O_2	↑ Osteoclasts	NC ↓	Not tested	Sanchez-de-Diego et al., 2021
BSO administration to mice	↑ H_2O_2 (via inhibition of GSH)	↑ Osteoclasts and Osteoblasts	NC ↓	Not tested	Almeida et al., 2007 Lean et al., 2003

NC = not changed.
[a] Age-dependent.
[b] Sex- and age-dependent.

2013) (Table 1). On the other hand, work performed using a different NOX4$^{-/-}$ mouse line led to inconsistent results with respect to the effects of NOX4 on bone mass (Chen et al., 2022; Watt et al., 2018). The contribution of NOX1 to bone homeostasis has not been described, so far. On the other hand, mice deficient in the cytoplasmic SOD1 have low osteoclast number in bone but osteoclast precursors from these mice display normal osteoclast differentiation in vitro (Nojiri et al., 2011). The authors propose that the decrease in osteoclasts is secondary to a deficient RANKL/M-CSF osteoclastogenic signaling due to impaired osteoblast viability. Together, this work provides some support for a role of cytoplasmic ROS produced by NOXs in osteoclastogenesis and bone resorption. However, due to the systemic nature of the genetic models used to examine the contribution of NOXs to bone homeostasis a direct role for these enzymes in osteoclasts and bone resorption in vivo remains to be elucidated.

Besides stimulating ROS production, RANKL promotes ROS accumulation by inhibiting the expression of antioxidants. The ratio of GSH/GSSG decreases during osteoclastogenesis, due to a decrease in total GSH (Huh et al., 2006). M-CSF and RANKL promote the accumulation of H_2O_2 in osteoclasts and their progenitors via FoxO-mediated transcription. FoxOs are a family of transcription factors that decrease ROS levels by upregulating the expression of antioxidant enzymes (de Keizer et al., 2011; Tothova et al., 2007). FoxO1, 3 and 4 are expressed in cells of the hematopoietic lineage and global combined deletion of the three factors increases the number of osteoclast progenitors in the bone marrow (Ambrogini et al., 2010; Tothova et al., 2007; Tsuchiya et al., 2013). Mice with conditional deletion of FoxO1, 3 and 4 in cells of the monocyte lineage (LysM-cre) have low bone mass due to an increase in osteoclast number (Bartell et al., 2014). The decrease in bone mass is associated with low catalase and high H_2O_2 levels and is mitigated by administration of the antioxidants catalase or *N*-acetylcysteine (NAC). In line with these findings, mice overexpressing FoxO3 in osteoclast lineage cells exhibit higher levels of catalase and lower H_2O_2 along with lower osteoclast number and high bone mass (Bartell et al., 2014). Moreover, expression of a constitutively active FoxO1 in osteoclast lineage cells causes severe osteopetrosis (Watanabe-Takano et al., 2021). These findings support the notion that a major role for FoxOs in osteoclastic cells is to stimulate antioxidant enzymes and suppress the ROS required for optimal osteoclast development. FoxO activity is regulated by the NAD-dependent Sirt1 deacetylase. Similar to FoxOs, Sirt1 in osteoclasts inhibits osteoclastogenesis and bone resorption (Almeida and Porter, 2019) (Fig. 2). In osteoclasts, deacetylation of FoxO1, 3 and 4 by Sirt1 stimulates FoxO activity and catalase expression (Kim et al., 2015). Accordingly, stimulation of Sirt1 with natural or synthetic compounds decreases osteoclastogenesis and attenuates the decrease in bone mass caused by aging, sex steroid deficiency or immobilization (Artsi et al., 2014; Mercken et al., 2014; Momken et al., 2011; Pearson et al., 2008; Su et al., 2007). However, whether the antioxidant role of Sirt1 in osteoclasts contributes to these effects remains unknown.

NRF2 is a transcription factor that promotes the expression of antioxidant and detoxification enzymes to maintain cellular redox homeostasis. KEAP1 (a cysteine thiol-rich detector of redox status) functions as a repressor of NRF2. Under oxidative distress, a de-repression mechanism releases NRF2 from KEAP1, causing activation of NRF2 (Yamamoto et al., 2018). Several laboratories have examined the skeleton of

mice with global deletion of NRF2. These studies led to inconsistent results with some indicating that NRF2 inhibits the accrual of bone mass while others found that NRF2 is required for normal accrual of bone mass, and others found no effects (Kim et al., 2014; Park et al., 2014; Rana et al., 2012) (Table 1). An extensive study by Pellegrini and colleagues examining both male and female NRF2 KO mice up to 15 months of age revealed a complex role for NRF2 in regulating bone mass that is dependent on sex and age (Pellegrini et al., 2017). This study also suggests that the expression of detoxifying enzymes in bone is dependent on NRF2 in females but not in males, supporting the idea that the mechanisms for the maintenance of redox homeostasis in bone are sex-specific. In line with this notion, mice with ablation of NRF2 in osteocytes or osteoblasts have low bone mass with marked sexual dimorphism (Sanchez-de-Diego et al., 2021). Interestingly, the changes in bone mass are attributed to increased bone resorption, suggesting that NRF2 in cells of the osteoblastic lineage indirectly suppresses osteoclast number. For a detailed description of the role of NRF2 in bone cells the readers are directed to a recent review on this topic (Han et al., 2022).

Overall, these findings indicate that the stimulation of mitochondrial and cytoplasmic ROS, as well as a suppression of antioxidant mechanisms are implicated in the pro-osteoclastogenic actions of RANKL. Moreover, the increase in ROS levels, in particular H_2O_2, is a critical and regulated step of osteoclastogenesis and not a mere consequence of the intrinsic role of macrophages in producing ROS or the elevated mitochondria number needed to support the high energy demands of bone resorption.

4.2. ROS in osteoblasts lineage cells

In contrast to osteoclast lineage cells and bone resorption, there is not much evidence to support a role for oxidative eustress in osteoblast lineage cells and bone formation. Work performed using osteoblast cell cultures suggests that Prdx2 negatively regulates BMP2-induced osteoblast differentiation (K.M. Kim et al., 2019). Prdx2 is a cytoplasmic protein and one of the six members of the peroxiredoxin family of enzymes which degrade H_2O_2. According to the effects seen on osteoblast differentiation, mice lacking Prxd2 have higher trabecular bone mass. However, the cellular causes of the changes in bone mass in the Prdx2 KO mice were not examined, so it remains unknown whether the number of osteoblasts is indeed increased in this model. Ucer and colleagues have examined the contribution of mitochondrial H_2O_2 in cells of the mesenchymal lineage to skeletal homeostasis using the mitoCAT transgene targeted to Prx1-Cre expressing cells, including stem and progenitor cells, stromal cells, osteoblasts, and osteocytes (Ucer et al., 2017). These mice display normal cortical and trabecular bone at a young age (up to 6 months). Mice with systemic mCAT overexpression have also been used to examine the role of H_2O_2 in skeletal homeostasis (Schreurs et al., 2020). These studies revealed that trabecular bone volume in the tibiae is slightly reduced in 16 wk-old mice, while no changes occur in vertebral bone. These effects, most likely reflect actions of mCAT in cells other than osteoclasts or osteoblasts.

The consequences of oxidative distress to the skeleton have been examined by multiple laboratories with genetic models of superoxide dismutase deletion, or pharmacologic administration of a glutathione reductase inhibitor such as L-buthionine-(*S*,*R*)-sulfoximine (BSO). Studies with a mouse model of SOD1 deletion indicate that cytoplasmic superoxide decreases BMD, causes aging-like changes in collagen cross-linking, and exacerbates the loss of bone mass that occurs with mechanical unloading (Morikawa et al., 2013; Nojiri et al., 2011). The surface areas of bone covered by osteoblasts and osteoclasts are decreased in the vertebrae of SOD1 KO mice, indicating low-turnover osteopenia. In vitro experiments with primary osteoblasts from these mice show that intracellular oxidative stress reduces proliferation and promotes cell death, providing an explanation for the decrease in osteoblast number in bone. Administration of BSO causes bone loss in mice in some studies but not others (Almeida et al., 2007; Lean et al., 2003). In contrast to the findings with the SOD1 KO mice, the decrease in bone mass with BSO is associated with high bone turnover (Lean et al., 2003).

Targeted effects of oxidative distress in mitochondria of cells of the osteoblast lineage have been examined with models of SOD2 depletion. Mice with deletion of SOD2 under the control of Dmp1-Cre or Runx2-Cre exhibit enhanced production of cellular superoxide in bone and low trabecular and cortical bone mass (Kobayashi et al., 2015; Schoppa et al., 2022). These changes are associated with a reduced number of osteoblasts and an increased number of osteoclasts. SOD2 loss also leads to a markedly disorganized osteocytic canaliculi network and a decrease in the number of live osteocytes (Kobayashi et al., 2015). Upregulation of Sost due to SOD2 deficiency has been proposed to contribute to the low bone mass phenotype. Bone from SOD2-deficient mice also exhibits a higher number of senescent cells but no changes in cell viability (Schoppa et al., 2022). Work performed with cultures of osteoblastic cells from SOD2 conditional KO mice supports the idea that elevated oxidant levels cause cellular senescence. These findings suggest that an increase in mitochondrial ROS in cells of the osteoblast lineage negatively impacts bone formation and, indirectly, stimulates bone resorption.

5. Oxidants in pathophysiological bone loss

Oxidative distress has been implicated in the biology of aging and aging-related diseases for many decades (Tan et al., 2018). Indeed, the free radical theory of aging initially described by Harman in the 1950s proposed that aging is caused by accumulation of damage inflicted by ROS (Harman, 1956). While many studies throughout the years have argued against the universal role of oxidative damage in aging, mitochondrial dysfunction and elevated ROS remain well-established hallmarks of aging (Giorgi et al., 2018; Lopez-Otin et al., 2013). ROS have also been implicated in other hallmarks of aging such as DNA damage and cellular senescence (Davalli et al., 2016; Lopez-Otin et al., 2013). Loss of redox homeostasis or the shift from oxidative eustress to distress underlies age-associated disease condition such as neurodegeneration and cardiovascular disease (Dai et al., 2017). Likewise, evidence from studies using pharmacological or genetic targets in mouse models indicate that H_2O_2 and perhaps other ROS contribute to the loss of bone mass with aging and estrogen deficiency, the two major causes of osteoporosis and fractures in humans.

5.1. Skeletal aging

Extensive evidence from animals and humans indicates that a decrease in osteoblasts and bone formation is a seminal mechanism of the decreased bone mass in old age (Manolagas and Parfitt, 2010). An increase in endocortical osteoclasts in long bones is also a major contributor to the loss of bone with age (Piemontese et al., 2017). In mice, skeletal aging is associated with increased levels of ROS and lipid peroxidation in bone along with other hallmarks of aging such as DNA damage and cellular senescence (Almeida et al., 2009; Farr and Almeida, 2018). Our laboratory has examined the contribution of mitochondrial H_2O_2 in cells of the mesenchymal or osteoclast lineage to skeletal aging (Ucer et al., 2017). In mice expressing the mitoCAT transgene targeted to the monocyte lineage the loss of bone mass with aging is similar to the controls, indicating that mitochondrial H_2O_2 in osteoclasts does not contribute to the increase in bone resorption and loss of bone mass with aging. In contrast, in mice with the mitoCAT transgene targeted to the mesenchymal lineage the decrease in bone formation markers and loss of bone mass with aging is attenuated (Fig. 3). Mice with systemic mCAT overexpression were used to examine the role of mitochondrial H_2O_2 to the bone loss that occurs with combined unloading and exposure to low grade ionizing radiation (to mimic space flight) (Schreurs et al., 2020). The transgene effectively diminished the levels of ROS in bone caused by the treatment. However, the loss of bone mass was indistinguishable between untreated and treated mice suggesting that an increase in

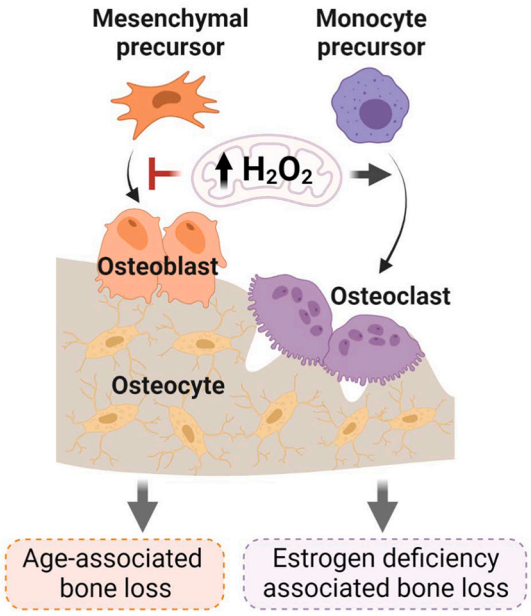

Fig. 3. Role of mitochondria H_2O_2 in bone pathophysiology. Studies performed with genetic models of targeted suppression of 11,202 in mitochondria have elucidated that H_2O_2 in osteoclastic cells contributes to the increase in osteoclast number and bone loss with estrogen deficiency, while an increase in H_2O_2 in mitochondria of mesenchymal cells contributes to the decrease in bone formation and bone mass caused by aging.

mitochondrial H_2O_2 is not sufficient to cause bone loss in this model of osteoporosis.

The contribution of oxidized phospholipids to bone physiology and skeletal aging has been examined with transgenic mice expressing a single-chain variable fragment (scFv) of the antigen-binding domain of E06 (Que et al., 2018). E06 is a natural IgM antibody which recognizes the phosphocholine moiety of oxidized phospholipids, but not native phospholipids. Young E06-scFv mice have high bone mass associated with decreased bone resorption and increased bone formation suggesting that lipid peroxidation has deleterious effects on bone remodeling under physiological conditions (Palmieri et al., 2021b). The E06 transgene also attenuates the age-associated trabecular bone loss in both male and female mice in line with the increase in lipid peroxidation with aging (Almeida et al., 2009; Barrera et al., 2018; Liu et al., 2013). E06-scFv mice were also protected against high-fat diet-induced bone loss but not against ovariectomy or unloading induced bone loss (Ambrogini et al., 2018; Palmieri et al., 2021a). These findings highlight the functional contribution of oxidized phospholipids to skeletal physiology and to some, but not all, causes of osteoporosis.

Numerous in vitro studies have elucidated cellular and molecular pathways responsible for the deleterious consequences of oxidative distress in osteoblast. Specifically, addition of H_2O_2, BSO, or 4-HNE to osteoblastic cell cultures has elucidated that these pro-oxidant compounds can inhibit cell proliferation and differentiation, and promote apoptosis and cellular senescence (Almeida et al., 2009; Almeida et al., 2007; Kim et al., 2020b). The effects on osteoblast proliferation and differentiation are due, at least in part, to the inhibition of critical pro-osteoblastogenic pathways such as the Wnt signaling (Almeida et al., 2009; Almeida et al., 2007). In line with the cellular effects of ROS in osteoblastic cells in vitro, the increase in ROS with aging in bone is associated with increased apoptosis of osteoblast and osteocytes, as well as with senescence of osteoblast progenitors, osteoblasts, and osteocytes (Almeida et al., 2007; Farr et al., 2016; Kim et al., 2017). To examine whether ROS contributes to the increase in osteoblast apoptosis with aging, Jilka et al. administered NAC and Catalase to aged mice and found that these antioxidants are effective in reducing ROS and increasing glutathione in bone (Jilka et al., 2010). Administration of antioxidants is also effective in decreasing osteoblast apoptosis indicating that osteoblast death is one of the consequences of the elevated ROS levels with aging.

Cellular senescence and dysfunctional mitochondria are intrinsically linked (Martini and Passos, 2022). Cellular senescence is a process in which cells stop dividing, evade apoptosis, and initiate a gene expression pattern known as the senescence associated secretory phenotype (SASP) (Gorgoulis et al., 2019; Lopez-Otin et al., 2013). Senescent cells also contain dysfunctional mitochondria that produce excessive ROS. Mitochondrial ROS in senescent cells can cause damage to DNA and other macromolecules (Martini and Passos, 2022). Besides autocrine effects, ROS produced by senescent cells can also exert paracrine effects in surrounding cells (Victorelli et al., 2019). Thus, senescent cells can damage neighboring healthy cells via both the SASP and ROS. Clearance of senescent cells attenuates age-associated dysfunction of multiple tissue. Several studies have used genetic and pharmacological approaches to examine the role of senescent cells in aged bone. Some studies, but not all, indicate that cellular senescence contributes to skeletal aging (Farr et al., 2017; H.N. Kim et al., 2019; Sharma et al., 2020). In any case, it remains unknown whether ROS contributes to the increase in osteoblastic cell senescence in aged bone. Overall, this work indicates that part of the mechanisms of aging leading to the loss of bone mass is an increase in mitochondrial H_2O_2 generation or a decline in the mechanisms that inactivate H_2O_2 in cells of the mesenchymal lineage.

5.2. *Bone loss with estrogen deficiency*

Estrogen deficiency causes loss of bone mass due to an increase in osteoclast number and excessive bone resorption (Almeida et al., 2017). Work performed with conditional deletion models of the estrogen receptor alpha (ERα) and ovariectomy (Manolagas et al., 2013; Vanderschueren et al., 2014) have functionally demonstrated that the protective effects of estrogens in females are mediated via the ERα in cells of the osteoclast lineage (Martin-Millan et al., 2010; Nakamura et al., 2007). Estrogens suppress the differentiation, activity and survival of osteoclasts via direct and indirect mechanisms (Almeida et al., 2017). Work by Lean et al. has implicated an increase in the generation of ROS in the pathologic bone resorption associated with estrogen deficiency (Lean et al., 2003). The increase in ROS is associated with a reduction in glutathione and thioredoxin, the major thiol antioxidants, as well as glutathione and thioredoxin reductases. Administration of the antioxidants NAC or ascorbate attenuates the ovariectomy-induced increase in osteoclasts and bone loss (Almeida et al., 2007; Lean et al., 2003) (Table 1).

The requirement for mitochondrial H_2O_2 in osteoclasts to the effect of ovariectomy in bone has been examined using mice with mitochondria-targeted catalase in monocyte lineage cells (Bartell et al., 2014; Ucer et al., 2017). The findings that these mice are protected from the loss of bone caused by estrogen deficiency indicates that mitochondria-derived H_2O_2 contributes to the pathologic bone resorption that occurs following loss of estrogens (Fig. 3). In contrast, the loss of bone mass with ovariectomy was easily seen in NOX4$^{-/-}$ mice (Chen et al., 2020), indicating that the ROS produced by NOX4 is not required for the increase in osteoclasts caused by estrogen deficiency. In line with the requirement of mitochondrial ROS to the increase in osteoclasts with estrogen deficiency, estrogen acting via ERα decreases the expression of several genes encoding components of mitochondria Complex I. Moreover, estrogen inhibits the stimulation of oxidative phosphorylation and ATP production by RANKL (Kim et al., 2020a). Overall, these findings indicate that attenuation of mitochondria activity and a reduction in mitochondrial ROS in osteoclastic cells contribute to the anti-resorptive effects of estrogens.

6. Overview

While much knowledge has been gathered in recent decades regarding the role of ROS is bone cells, many questions remain unanswered. Multiple lines of evidence indicate that there are fundamental differences in the way osteoblasts and osteoclasts handle ROS. Oxidative eustress is important for optimal osteoclast development, however the specific molecular targets of H_2O_2 in osteoclasts remain unknown. Estrogens decrease the level of H_2O_2 associated with physiological osteoclast development. It was proposed that estrogens could stimulate the expression of antioxidant enzymes (Lean et al., 2003; Manolagas, 2010) and more recently that estrogens attenuate RANKL-stimulated mitochondria activity (Kim et al., 2020a). However, more work is needed to elucidate the mechanisms responsible for the antioxidant effect of estrogens. In contrast to osteoclasts, physiological levels of mitochondrial H_2O_2 in osteoblastic cells do not overtly impact the skeleton in young mice. On the other hand, mitochondria H_2O_2 in cells of mesenchymal lineage (Prx1-Cre) contribute to skeletal aging. Nonetheless, because Prx1-Cre causes recombination in mesenchymal progenitors and all their descendants it remains unclear which cell type is the target of the protective effects of mitoCAT on bone formation. Multiple cells in aged mice such as osteoblast progenitors, osteoblast, and osteocytes display markers of damage (Farr et al., 2016; Kim et al., 2017). Yet, it remains unknown whether these markers are functionally related to the loss of bone mass with age. It has been proposed that an increase in ROS and lipid oxidation with aging could cause osteoblast apoptosis and a diversion of precursor cells towards the adipocyte instead of osteoblast lineage. However, mice lacking BAX and BAK, the indispensable proteins for mitochondria mediated apoptosis, in osteoblastic lineage cells are not protected against age-related bone loss (Jilka et al., 2014). Likewise, mice lacking marrow adipocytes due to deletion of PPARγ in the mesenchymal cell lineage, are not protected against skeletal aging (Almeida et al., 2020). It remains possible that an increase in mitochondrial H_2O_2 promotes cellular senescence, which contributes to age-related bone loss.

In conclusion, studies with pre-clinical models suggest that attenuation of oxidative eustress and distress in bone cells could combat the loss of bone mass with estrogen deficiency and aging. The critical role of mitochondrial ROS and mitochondrial oxidative damage in different diseases has prompted the use of mitochondrial-targeted antioxidant therapies as likely treatments (Amorim et al., 2022). The best-characterized mitochondria targeted antioxidant, MitoQ, has been shown to protect against oxidative distress and alleviate many diseases of age in mice (James et al., 2007; Murphy, 2016; Shinn and Lagalwar, 2021). Importantly, in clinical studies MitoQ has been shown to combat vascular aging in older adults (60–79 years) (Rossman et al., 2018). Nonetheless, despite the progress achieved over the last decades, combatting oxidative distress in age-associated diseases has proven challenging, due to the complex role of oxidants in physiology and pathophysiology. Recent findings obtained following a multi tissue (bone not included) organismal-level analysis of the cysteine oxidation proteome in young and old mice revealed that the global cysteine modification do not increase with age, in contrast to what could be anticipated due to the increase in ROS (Xiao et al., 2020). Instead, distinct redox signaling networks are selectively altered in every tissue. Thus, a better understanding of mechanisms of redox regulation in skeletal physiology and disease is warranted.

Funding

The authors' research is supported by the National Institutes of Health (R01AG068449, R56AR056679, R01AR080736, P20GM125503) and the UAMS Bone and Joint Initiative. A. Marques-Carvalho is supported by a doctoral research fellowship from the European Regional Development Fund (ERDF), through Portuguese national funds via *Fundação para a Ciência e Tecnologia* (FCT) (SFRH/BD/140817/2018).

CRediT authorship contribution statement

Maria Almeida: Conceptualization, supervision, writing. **Adriana Marques Carvalho**: Original draft preparation, figures and table preparation. **Ha-Neui Kim**: Figure preparation, review of published work, writing. **All authors** revised and approved the final version.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

Acknowledgements

The illustrations were prepared using BioRender. We apologize for not citing other relevant work due to space constrains.

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