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Reactive Oxygen Species, Aging and Articular Cartilage Homeostasis

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Abstract

Chondrocytes are responsible for the maintenance of the articular cartilage. A loss of homeostasis in cartilage contributes to the development of osteoarthritis (OA) when the synthetic capacity of chondrocytes is overwhelmed by processes that promote matrix degradation. There is evidence for an age-related imbalance in reactive oxygen species (ROS) production relative to the anti-oxidant capacity of chondrocytes that plays a role in cartilage degradation as well as chondrocyte cell death. The ROS produced by chondrocytes that have received the most attention include superoxide, hydrogen peroxide, the reactive nitrogen species nitric oxide, and the nitric oxide derived product peroxynitrite. Excess levels of these ROS not only cause oxidative-damage but, perhaps more importantly, cause a disruption in cell signaling pathways that are redox-regulated, including Akt and MAP kinase signaling. Age-related mitochondrial dysfunction and reduced activity of the mitochondrial superoxide dismutase (SOD2) are associated with an increase in mitochondrial-derived ROS and are in part responsible for the increase in chondrocyte ROS with age. Peroxiredoxins (Prxs) are a key family of peroxidases responsible for removal of H_2O_2, as well as for regulating redox-signaling events. Prxs are inactivated by hyperoxidation. An age-related increase in chondrocyte Prx hyperoxidation and an increase in OA cartilage has been noted. The finding in mice that deletion of SOD2 or the anti-oxidant gene transcriptional regulator nuclear factor-erythroid 2-related factor (Nrf2) result in more severe OA, while overexpression or treatment with mitochondrial targeted anti-oxidants reduces OA, further support a role for excessive ROS in the pathogenesis of OA. Therefore, new therapeutic strategies targeting specific anti-oxidant systems including mitochondrial ROS may be of value in reducing the progression of age-related OA.

Graphical Abstract:

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The authors declare no-conflicts of interest

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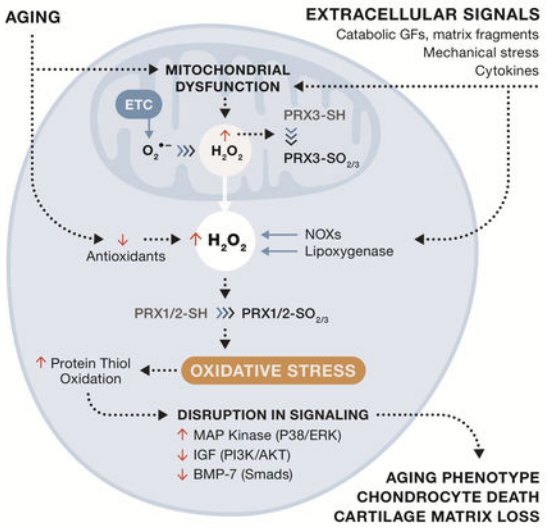

Keywords

Aging; cell signaling; reactive oxygen species; chondrocytes; cartilage; osteoarthritis

1. Introduction

Articular cartilage homeostasis can be defined as the state at which degradation of extracellular matrix components is balanced by synthesis. In normal, healthy, adult articular cartilage the resident chondrocytes are quiescent, exhibiting low metabolic activity and very little turnover of matrix components is present. Because articular cartilage lacks a blood supply, the cells exist in a relatively hypoxic environment. Although the precise oxygen tension in cartilage *in vivo* is not known, the best estimates place it above 1% and most likely around 5% with a decreasing gradient from the cartilage surface to the deeper zones [1]. Despite living in a low O_2 environment, chondrocytes have abundant and active mitochondria that contribute to ATP production [2]. Disruption of mitochondrial function resulting in increased levels of intracellular reactive oxygen species (ROS) has been hypothesized to disrupt cartilage homeostasis and contribute to cartilage damage seen in osteoarthritis (OA) [2, 3].

OA is the most common form of arthritis affecting over 30 million adults in the United States [4] with hip and knee OA representing one of the leading causes of disability globally [5]. Age is a primary risk factor for OA and aging changes in joint tissues, including the articular cartilage, contribute to the development and progression of OA [6]. Although OA affects all the tissues of the articular joint, degradation and loss of articular cartilage is a central feature [7]. Cartilage degradation in OA results from a disruption in homeostasis due to activation of the chondrocytes by various factors that promote production of matrix degrading enzymes in excess of the capacity of the chondrocyte to replace damaged and degraded matrix components.

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The factors that activate chondrocytes to promote matrix degradation include excessive and abnormal mechanical loading, pro-inflammatory cytokines and chemokines, as well as Wnt ligands and factors activating the innate immune system [7–9]. As matrix degradation begins, it is propagated by a feed-forward loop mediated by matrix fragments and other damage-associated molecular pattern molecules (DAMPs) generated in the cartilage and neighboring joint tissues including the synovium. Important to this review, many of these OA factors stimulate chondrocytes to produce ROS and utilize ROS as second messengers in mediating intracellular signaling events that regulate downstream gene expression including expression of matrix degrading enzymes [10, 11]. Here, we will review the various types and sources of ROS produced by chondrocytes and the mechanisms by which they disrupt cartilage homeostasis including the role that aging plays in this process.

2. Chondrocyte Reactive Oxygen Species: Types, Sources, and Removal

The types of ROS best characterized in chondrocytes and in the context of cartilage homeostasis, aging and osteoarthritis are superoxide ($O_2\cdot$) and hydrogen peroxide (H_2O_2). Although more accurately considered as reactive nitrogen species (RNS), ·NO and ·NO derived products such as peroxynitrite ($ONOO^-$) are also found in cartilage and regulate chondrocyte function. While other ROS exist, for the purposes of this review we will cover these main players, including how and where they are generated and how they are removed as well as their role in cartilage homeostasis.

2.1. Superoxide and Superoxide Dismutase

$O_2\cdot$ is generated mainly from two different sources: incomplete oxidative phosphorylation in the mitochondria and non-mitochondrial membrane-bound nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (Fig. 1) [12, 13]. $O_2\cdot$ is a potent ROS that has a powerful influence on the intracellular redox state. It is essential for a healthy immune response, but moreover, it can directly oxidize proteins, which in turn, can dramatically shift signal transduction, gene expression, and cell cycling. Superoxide dismutase (SOD) catalyzes the conversion of $O_2\cdot$ to H_2O_2. SODs can also generate the more reactive and damaging ROS, the hydroxyl radical (OH·), by converting $O_2\cdot$ to OH· in the presence of Fenton-reactive metals. Since it is such a highly reactive species with a cellular half-life of about 10^{-9}s, OH· has limited time to induce damage unless it is being continuously generated. H_2O_2 is less detrimental than $O_2\cdot$ and OH·, but it is present longer (cellular half-life of about 1ms) before it undergoes further reduction by enzymes including peroxiredoxin and catalase (detailed below) [14].

In the mitochondrial matrix, the concentration of $O_2\cdot$ has been estimated to be in the range of 10–200 pm [15]. With age, mitochondrial function decreases resulting in increased basal production of $O_2\cdot$ and subsequent increases in oxidative stress [16]. Moreover, it has been reported that mechanical loading of chondrocytes *in vivo* promotes mitochondrial generation of $O_2\cdot$ accompanied by a decrease in the expression of the mitochondrial SOD, SOD2 [17]. Thus, the age-related changes that promote $O_2\cdot$ production combined with $O_2\cdot$ stimulated by excessive mechanical loading and a decrease in SOD2 could exacerbate the hallmark redox imbalance linked with OA pathogenesis.

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The NADPH oxidase (Nox) enzyme family is a key enzymatic source of $O_2\cdot$ as well as H_2O_2. There are seven Nox family members that include Nox1–5 and dual oxidases (Duox)1–2. All Nox family members produce $O_2\cdot$ while Nox4 and the Duoxs can generate H_2O_2 in addition to $O_2\cdot$ [18]. Noxs have a firmly established importance in immune function, due to Nox presence and participation in macrophage phagocytosis and neutrophil bactericidal activity. The Noxs, when activated in immune cells, generate a "respiratory burst" of $O_2\cdot$ meant to aid in the killing response against bacterial invasion [19–21]. However, it is also becoming increasingly clear that $O_2\cdot$ and H_2O_2 produced by Noxs in many cell types are critical for normal signal transduction stimulated by a host of growth factors and cytokines [22]. There is evidence that ROS produced by Nox2 and Nox4 are involved in chondrocyte differentiation [23] although Nox2 has also been implicated in chondrocyte cell death mediated by IL-1β [24] and Nox4 in IL-1β stimulation of matrix metalloproteinase 1 expression [25].

In OA cartilage, Nox4 is thought to be the predominant isoform active in chondrocytes where activation of Nox4 by proinflammatory cytokine stimulation increases $O_2\cdot$ and H_2O_2 production [26, 27]. In turn, increased activation of Nox4 and resultant ROS generation can exacerbate cartilage breakdown by influencing signaling events responsible for MMP production [27].

In addition to increased $O_2\cdot$ and H_2O_2 production, redox imbalance in OA cartilage is also due to decreased levels of anti-oxidant enzymes that include SOD. SOD expression has been examined in articular cartilage where all three SOD enzymes (SOD 1, 2, 3) were found to be present [28, 29]. Compared to normal cartilage, a gene array study found SOD2 and SOD3, as well as glutathione peroxidase, were down-regulated in human OA cartilage [30]. Examination of cartilage from Dunkin Hartley guinea pigs that develop spontaneous OA with age also revealed a reduction in SOD2 that was associated with OA progression [28]. In the same study, the SOD2 promoter was found to be methylated in human OA chondrocytes to a greater degree than cells from normal cartilage which would be expected to result in decreased SOD2 expression. Depletion of SOD2 by siRNA in human chondrocytes increased levels of ROS detected using the sensor MitoSOXTMRed but unexpectedly resulted in decreased matrix metalloproteinase (MMP) expression, suggesting a negative feedback role for superoxide in chondrocyte MMP production [28].

In an aging study using cartilage isolated from rats between 10 and 30 months of age, an increase in SOD2 protein levels was noted but SOD2 activity declined [31]. The decline in activity was associated with an increase in SOD2 acetylation due to a decline in Sirt3. Mice with Sirt3 deletion were found to develop more severe age-related OA changes when compared to controls suggesting that an age-related loss in Sirt3 could result in increased SOD2 acetylation and decreased SOD2 activity contributing to age-related OA [31]. An OA associated decrease in SOD3 (the extracellular SOD) has also been noted in human cartilage and in the STR/ort mouse model of spontaneous OA where it was associated with increased immunostaining for nitrotyrosine as a marker of oxidative damage [29]. These studies support the notion that decreased levels of SOD2 and/or SOD3 can contribute to age-related OA but the precise mechanisms by which they do so require further investigation.

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2.2. Hydrogen Peroxide and Peroxidases

H_2O_2 is a derivative of $O_2\cdot$. $O_2\cdot$, generated either via incomplete/inefficient reduction of molecular oxygen in the mitochondrial electron transport chain and/or through Nox activity, is acted upon by SOD enzymes and converted to H_2O_2 (Fig.1). Notably, Nox4 has been shown to additionally produce H_2O_2 directly, unlike the other isoforms that only produce $O_2\cdot$ [19, 26]. Xanthine oxidase (XO), an enzyme that oxidizes hypoxanthine to xanthine, is an additional source of H_2O_2, which seemingly contributes to inflammation and degradation of cartilage. XO appears to make this contribution more through its activity in synovial membranes rather than in the chondrocytes themselves [32, 33]. Activation of these H_2O_2-producing enzymes occurs through a variety of different stimuli, as discussed above, including stimulation of Noxs by pro-inflammatory cytokines. H_2O_2 is freely diffusible and is an important mediator for intra- and intercellular signaling, for both stress and non-stress responses (discussed further below in section 6).

2.2.1. Peroxiredoxins—Peroxiredoxins (Prxs) are a family of antioxidant proteins predominantly responsible for the reduction/detoxification of H_2O_2 (Fig.1). Prxs are also able to reduce organic hydroperoxides (ROOH) and peroxynitrite [34]. Due to their catalytic rates of $10^6 - 10^7\ M^{-1}s^{-1}$ and high abundance (0.1–1% of total soluble protein), Prxs are considered the first line in detoxification, outcompeting other peroxidases like catalase and glutathione peroxidase making Prxs responsible for reducing around 90% of cellular peroxides [35, 36]. Their ubiquitous expression in all tissues, including cartilage [37, 38], make them important in aging and cartilage homeostasis. There are six Prxs in humans. Prx1 and Prx2 are in the cytosol, Prx3 in the mitochondria, and Prx4 in the endoplasmic reticulum. Prx5 has a broad subcellular localization. It has been detected in mitochondria, peroxisomes, the nucleus, and the cytosol. Prx6 is mainly located in the cytosol, but sometimes is also associated within membranes [34].

Prxs are divided into three classes depending on the number of cysteine (Cys) residues and whether they form inter- or intramolecular disulfide bonds during catalysis [39]. All Prxs have a conserved cysteine in the active site, referred to as the peroxidatic cysteine (Cys-S_P or C_P) [40]. In humans, Prx1–4 are the typical, 2-Cys Prxs, meaning they contain an additional cysteine called the resolving cysteine (Cys-S_R or C_R) usually located near the C-terminus allowing for the formation of an intersubunit disulfide bond between a C_P and C_R of adjacent monomers during catalysis [41]. Prx5 is an atypical, 2-Cys Prx, meaning it contains a C_R, but it is located in a different position. However, the C_P location still allows for intersubunit disulfide bond formation [42]. Prx6 is a 1-Cys Prx, so the C_R is replaced by a thiol-containing reductant [43]. The different Prxs progress through catalysis of H_2O_2 the same way, despite the differences regarding C_R [39, 41, 44].

The reduced C_P interacts with a molecule of H_2O_2 and becomes oxidized to the sulfenic acid species (C_P-OH) whereas the H_2O_2 is reduced to water (Fig.1). At this point two things can happen depending on the amount of H_2O_2 locally present. One route is the C_P-OH can rapidly condense with the C_R (regardless of source of C_R depending on subclass of Prx) and form an inter- or intramolecular disulfide bond which can then be reduced by the NADPH-dependent thioredoxin (Trx)/thioredoxin reductase (TrxR) system which returns the Prx to a

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catalytically active status. The other option is the C_P-OH can undergo up to two additional rounds of catalysis and become hyperoxidized (C_P-$O_{2/3}$). The hyperoxidized Prx is inactivated as a peroxidase but the inactivation is reversible by the ATP-dependent sulfiredoxin (Srx) enzyme [34]. Although publications on sulfiredoxin in the context of cartilage homeostasis are lacking, Trx has been shown to be expressed in cartilage [45] and overexpression in Trx transgenic mice was protective in a model of inflammatory arthritis [46].

The human Prxs are variably sensitive to hyperoxidation, which is a cellular means of differentiating and fine-tuning signaling activation responses to stress and non-stress stimuli. For instance, Prx1 differs from Prx2, thus, despite their high sequence similarity and co-localization in the cytosol, their functions are non-redundant [36, 47, 48]. One example of this is that Prx2 has been shown to hand off oxidative equivalents to STAT3 in a redox relay whereas Prx1 interacts with ASK1 [49]. Additionally, Prx1 activity is regulated more readily by phosphorylation than Prx2, also, further supporting their unique cellular roles [50, 51]. Interestingly, higher basal hyperoxidation of Prx1–3 was observed in older human chondrocytes, which was associated with inhibition of pro-survival Akt signaling and activation of apoptotic p38 signaling [38] (further discussed in section 7 below).

2.2.2. Catalase—Catalase is another peroxidase enzyme that catalyzes the reduction of H_2O_2 to water. It is chiefly localized to the peroxisome but has been detected in heart mitochondria. It has been reported that induced mitochondrial expression of catalase (MCAT) in transgenic mice resulted in lifespan extension, but induced nuclear expression or peroxisomal overexpression did not [52]. MCAT mice displayed reduced age-related oxidative damage and had approximately 20% longer lifespans. In OA patients, it has been shown that red blood cell catalase activity is increased in comparison to normal controls [53]. While this could suggest catalase plays a pathogenic role in OA, the evidence taken together supports catalase playing a compensatory, protective role. For example, Collins et al.[38] described in human chondrocytes and transgenic MCAT mouse cartilage how mitochondrial expression of catalase attenuated ROS-induced Prx oxidation/hyperoxidation. This ultimately resulted in increased cell survival in chondrocytes and reduced OA severity in aged mice.

2.2.3. Glutathione/ Glutathione peroxidase/Glutathione reductase system—Gluthathione, a highly abundant thiol found in the cytosol, mitochondria, and nucleus, can be either reduced (GSH) or oxidized (GSSG). Decreased GSH levels are associated with reduced synthesis of cartilage extracellular matrix components including proteoglycan and hyaluronic acid [54, 55]. GSH is converted to GSSG by glutathione peroxidase (Gpx) through an enzymatic mechanism that requires hydrogen peroxide-mediated oxidation of the Gpx selenocysteine residue. In order to complete the cycle, glutathione reductase (GR) then reduces the GSSG back to GSH in an NADPH-dependent fashion. In some eukaryotic tissues, decreases with age in GSH have been reported [56, 57]. In chondrocytes, GSH levels showed no change with age, but the GSSG levels were >4-fold higher in older chondrocytes representing redox imbalance indicative of higher oxidative stress [58]. In that study, higher levels of GSH, which could be experimentally achieved via treatment with thiol antioxidants

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or modification of culture conditions, were associated with increased chondrocyte survival. Increased levels of oxidized glutathione have also been observed in bovine cartilage in response to mechanical loading [59] while Gpx was noted to be downregulated in human OA cartilage [30]. With age and age-related increases in oxidative stress, the glutathione system can be imbalanced from many different directions and can contribute to pathogenesis, indicating further studies in cartilage and OA are warranted.

2.3. Nitric Oxide

·NO is generated by three different ·NO synthase (NOS) enzymes: inducible NOS (iNOS), endothelial NOS (eNOS), and neuronal NOS (nNOS). NOS enzymes catalyze the NADPH-dependent, two-step process wherein L-arginine is oxidized and converted to L-citrulline [60] (Fig. 2). All three isoforms of NOS are detected in chondrocytes although most studies have focused on iNOS which is upregulated by pro-inflammatory factors [61–63]. In the context of cartilage, studies have suggested that ·NO can promote chondrocyte death via apoptosis [61], while others have shown that ·NO alone does not cause chondrocyte death but rather NO induces apoptosis when it reacts with $O_2\cdot$ to form the highly oxidizing ONOO- [64]. ·NO has also been suggested to reduce proteoglycan synthesis and alter cell-matrix interactions mediated by binding of fibronectin to the α5β1 integrin through disruption of the intracellular signaling complex that contains focal adhesion kinase, actin, and RhoA [65]. Proteoglycans are an important cartilage matrix component responsible for the resiliency of cartilage and so inhibition of synthesis mediated by ·NO has the potential to disrupt cartilage homeostasis.

·NO production via activation of iNOS is initiated by signaling from pro-inflammatory cytokines including IL-1β, IL-17, TNF-α, and IFNγ [60]. There is some evidence that with increasing age, ·NO production by iNOS is less responsive to some activators, including IL-1β [66]. In chondrocytes isolated from OA cartilage, iNOS expression, activity, and levels of the downstream product ·NO are increased, which perpetuates inflammatory cytokine effects via a positive feedback loop [67]. This ultimately causes more cartilage degradation, and if sustained over time, could trigger apoptosis [61]. Cartilage degradation products like fibronectin fragments (FN-f), which notably accumulate in the cartilage and synovial fluid in osteoarthritis (OA), have been shown *in vitro* to increase ·NO production through stimulation of iNOS in human chondrocytes [68, 69]. Moreover, iNOS knockout mice have increased protection against experimental OA, presumably because of lower ·NO concentrations [70]. These studies suggested iNOS might be a suitable therapeutic target to reduce cartilage destruction in OA. However, a large randomized clinical trial in participants with knee OA found that iNOS inhibition was ineffective in slowing OA progression [71].

·NO production via endothelial NOS (eNOS) is relatively low, and in contrast to iNOS activation, eNOS activation is dependent on calcium and calmodulin concentrations [72]. Thus, because of force-associated influxes of Ca^{2+}, mechanical influences like sheer stress and static and intermittent compression can activate eNOS and increase ·NO generation [73]. This is particularly relevant in cartilage because of the load bearing nature of the joint and injury-based destabilization that can change these mechanical influences and resultant ·NO production. Furthermore, phenotypes of eNOS-deficient mice highlighted the unique role

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and importance of eNOS in cartilage. The mice showed reduced chondrocyte proliferation in the growth plate and endochondral bone growth as well as premature prehypertrophic chondrocyte differentiation during cartilage development [74]

Regarding nNOS, it was originally characterized as being expressed mainly in neuronal tissue, so requirements for nNOS expressed in cartilage have not been well studied. The relative levels of ·NO produced by nNOS in chondrocytes is not known, although nNOS expression was noted in normal chondrocytes while iNOS was suggested to be the dominant isoform in OA chondrocytes [63, 75]. One study found that chemical nNOS inhibition promoted cartilage restoration in a rat model of cartilage damage whereas iNOS inhibition had no effect [62]. Further work is needed to better define the role of nNOS in cartilage homeostasis.

Once generated, ·NO, which is considered a gas, diffuses freely across membranes, making it a potential messenger for both paracrine and autocrine signaling. For redox functions, ·NO can react with the enzyme metal cofactors like iron, copper, and zinc to regulate enzyme activity. For example, ·NO inhibits copper-containing cytochrome P-450 and iron-containing catalase disrupting the redox balance and causing an increase in $O_2\cdot$ and H_2O_2 production [60, 61, 76–78]. It has also been established that by binding to a reactive cysteine thiol, ·NO can form S-nitrosothiols, a type of post-translational oxidative modification.

NO derivative products comprise a family of RNS that can act with ROS to cause macromolecular cellular damage and nitrosative stress. For example, $O_2\cdot$ reacting with ·NO produces $ONOO^-$ which leads to nitrotyrosine generation, another type of post-translational modification indicative of oxidative stress and damage. Nitrotyrosine presence is related to aging and age-related diseases, including osteoarthritis [58]. Peroxynitrite can be protonated to form peroxynitrous acid (ONOOH), which is a compound extremely detrimental to the cell. ONOOH decomposes to NO_2 and hydroxide ion (OH-), both of which can further react [60, 77, 78]. In addition to the production of nitrotyrosine and the depletion of –SH groups, ONOOH oxidizes lipids, causes DNA strand breakage, and deamination of DNA bases. Sequestering ·NO production through NOS inhibition and/or scavenging $O_2\cdot$, through many different therapeutic approaches including antioxidant supplements, drugs, dietary polyphenols, and herbal medicines, could inhibit the deleterious effects associated with RNS [76]. However, the role ·NO plays in OA is a multi-faceted, complicated one warranting more examination and understanding.

3. Mitochondrial oxidative stress and redox signaling in cartilage and aging

Mitochondrial function declines with age in a variety of tissues and cell types and mitochondrial dysfunction is widely viewed as a ‘hallmark of aging’ [79]. The mitochondria are a key source of ROS. The ‘free radical theory of aging’ states that age-related mitochondrial dysfunction leads to increased generation of ROS and subsequent oxidative stress conditions that cause cellular damage to promote the aging phenotype and the development and progression of age-related diseases [80, 81]. Although this theory has received widespread attention, current data suggests that age-associated alterations in

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mitochondrial antioxidant capacity and increased oxidative stress may specifically lead to disturbances in physiological cell signal transduction to compromise cellular integrity and promote aging processes [81, 82]. Increasing evidence suggests that age-related oxidative stress related to mitochondrial dysfunction could promote a loss of homeostasis in cartilage and contribute to the development of OA (Fig.3).

Depletion of mitochondrial superoxide dismutase 2 (SOD2) leads to increased mitochondrial DNA strand breaks and mitochondrial dysfunction in chondrocytes [83] and significantly enhances cartilage degeneration in a post-traumatic mouse model of OA [17]. As noted above in section 2.1, an age-associated reduction in SOD2 activity due to increased lysine acetylation has been observed that may contribute to the development of OA [31]. The finding that SOD2 activity was increased when aged chondrocyte homogenates were incubated with SIRT3 and its cofactor NAD^+, suggests that strategies aimed at maintaining SIRT3 levels in aging cartilage could represent a therapeutic strategy to sustain SOD2 activity, an effect which could considerably alleviate age-related mitochondrial dysfunction and redox imbalance.

Farnaghi et al [84] have recently demonstrated the effect of MitoTEMPO, an antioxidant targeted to the mitochondria, to reduce OA severity and restore cartilage integrity in two models of cholesterol-induced OA. Immunohistochemical analysis showed that MitoTEMPO treated animals displayed lower levels of the chondrocyte hypertrophy marker, type X collagen (COL10), lower markers of cartilage matrix degradation measured using antibodies to cleaved aggrecan (NITEGE and DIPEN) and presented with less cell death than control treated animals [84]. Complimentary *in vitro* analyses demonstrated that treatment of chondrocyte pellets with MitoTEMPO significantly reduced cholesterol-induced proteoglycan loss, increased aggrecan expression, and reduced *COL10* and *MMP13* gene expression compared to controls. In addition, chondrocyte monolayer cultures were treated with cholesterol in the presence or absence of MitoTEMPO. Treatment with MitoTEMPO reduced cholesterol-induced ROS production, maintained ATP synthesis and preserved mitochondrial membrane potentials [84].

Expression of catalase targeted to the mitochondria (MCAT) has previously been shown to extend longevity in mice and attenuate various markers of age-associated diseases in a variety of tissues and organisms, in part by preventing age-related mitochondrial dysfunction and restoring mitochondrial redox balance [85]. In the context of cartilage, adenoviral expression of MCAT has been shown to abrogate mitochondrial Prx3 hyperoxidation, reduce catabolic MAP kinase signaling and restore pro-survival Akt signaling in primary human chondrocytes treated with the H_2O_2-generating, redox cycling oxidant, menadione [38]. Femoral cap explants derived from MCAT transgenic mice displayed decreased menadione-induced global Prx hyperoxidation and reduced catabolic p38 signaling compared to menadione-treated wild-type control femoral caps. Of particular note, a reduction in the severity of age-related OA was observed in aged (18–24 month old) MCAT transgenic mice when compared to wild-type controls [38]. Taken together, these findings suggest that age associated mitochondrial dysfunction and redox imbalance may contribute to aberrant intracellular signal transduction and contribute to the aging phenotype in cartilage.

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4. Mitochondrial DNA haplogroups influence redox status in aging and OA

Increasing evidence implicates a role for mitochondrial DNA (mtDNA) haplogroups in the pathogenesis of aging and age-related diseases, including OA [86]. For example, in the context of aging, individuals carrying haplogroup J have been more frequently observed in aging populations suggesting that this subset of the population may have an advantageous polymorphism towards extended longevity [87, 88]. This correlates with an association between individuals carrying mtDNA haplotype J and a reduced risk of knee [89, 90] and hip OA [91], when compared to other mtDNA haplogroups, but the underlying mechanisms behind these observations remain to be elucidated.

A series of studies analyzing OA biomarkers in the serum of OA and non-OA subjects shows that OA patients carrying haplogroup H display an increase in the cartilage degradative enzyme, MMP-3, when compared to OA patients carrying haplogroup J [92]. In the absence of OA, individuals carrying mtDNA haplogroup H also displayed enhanced MMP-13 serum levels [92] and trended towards reduced levels of the H_2O_2 detoxifying antioxidant, catalase [93]. In another study, OA and non-OA subjects harboring haplogroup H displayed increased serum levels of molecular biomarkers that indicate collagen type II cleavage and degradation, when compared with OA and non-OA subjects carrying haplogroup J [92]. Interestingly, OA patients carrying haplogroup H presented with enhanced serum levels of nitrated type II collagen, suggesting that haplogroup H may generate enhanced levels of oxidative stress [92], which may contribute to the enhanced collagen degradation observed in these patients.

Indeed, it has been suggested that differences in mitochondrial function and redox homeostasis between mtDNA haplogroups may contribute to OA susceptibility and the degenerative processes involved in aging [86, 89, 94]. For example, Fernandez-Moreno et al [89] demonstrated significant differences in mitochondrial metabolism, ROS generation and cell survival in transmitochondrial cybrids containing haplogroup J or H. Mitochondrial respiration was significantly lower in cybrids containing haplogroup J which correlated with a decrease in ATP production, when compared to H containing cybrids. Importantly, J cybrids displayed reduced levels of peroxide/ peroxynitrite formation, decreased superoxide anion levels and were less susceptible to oxidative stress mediated cell death, than H cybrids [89]. Taken together, it appears that aging and OA prevalence are associated with mtDNA haplogroups. The protective effects of particular mtDNA haplogroups appears, at least in part, to be associated with enhanced mitochondrial function and redox balance.

5. ROS-induced cell death in cartilage

An age-related reduction in cartilage cellularity has been proposed to be a mechanism contributing to OA pathogenesis but a causal relationship between chondrocyte cell death and cartilage damage remains unclear [95–97]. Several studies suggest that enhanced oxidative stress conditions, that are implicit with aging, can stimulate pro-death cell signaling pathways to compromise chondrocyte integrity and promote cartilage damage [38, 98, 99]. Although the pathways activated by ROS appear diverse, mitochondrially generated ROS may play a key role in activating cell signaling pathways to promote cell death. For

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example, mitochondrial H2O2 generated by menadione has been shown to activate MKK3/6-p38 signaling to induce rapid cell death in human chondrocytes [38]. Adenoviral expression of mitochondrial catalase was associated with enhanced pro-survival Akt activation and phosphorylation of BAD (ser136) as well as decreased MKK3/6-p38 signaling which maintained survival. Pharmacological inhibition of p38 was also shown to maintain cell viability in response to H_2O_2 demonstrating that chondrocyte cell death was, in part, dependent on the p38 MAP kinase signaling pathway [38].

Additionally, tert*butyl*-hydroperoxide (TBHP) has been shown to promote the collapse of the mitochondrial membrane, increase caspase and PARP cleavage and increase markers of mitochondrial apoptosis such as Bax and cytochrome c levels to promote cell death in chondrocytes [100]. In that study, pretreatment with trehalose, an activator of autophagy, reduced TBHP-induced mitochondrial dysfunction and protected against cell death and trehalose reduced the severity of surgically-induced OA in mice. Immunohistochemical analyses showed that trehalose receiving mice exhibited lower caspase 3 and cytochrome c levels and a decrease in TUNEL positive cells, indicating reduced cell death when compared to controls, indicating that reducing cell death through suppression of oxidative stress-induced mitochondrial dysfunction and promotion of autophagy may be beneficial in OA.

In other studies, overexpression of the redox sensitive DNA repair enzyme hOGG1 was shown to reduce IL-1β and TNF-α-induced mitochondrial superoxide production and decrease mitochondrial DNA damage to maintain cell viability in chondrocytes derived from OA donors [101]. ROS dependent activation of protein kinase C (PKC-β1) has also been shown to induce chondrocyte cell death, an effect which was abolished by inhibiting mitochondrial electron transport chain complex V, by addition of the superoxide scavenger, Tiron, or with specific inhibitors of PKC-β1 [102]. Collectively, these data demonstrate the ability of various ROS to act as secondary messengers to stimulate a plethora of cell signal transduction pathways in chondrocytes to promote cell death. Targeting antioxidant systems or inhibiting specific redox sensitive proteins that are implicated in pro-death pathways may be useful in maintaining cellular survival and promoting cartilage integrity.

6. Cellular senescence in aging and OA

In addition to mitochondrial dysfunction, cellular senescence represents another ‘hallmark of aging’ [103]. Cellular senescence is characterized by the cell entering a state of permanent proliferative arrest, in response to various signaling cues, whilst maintaining metabolic activity [104]. A role for cellular senescence in aging is well documented in a variety of tissues and may contribute to the pathogenesis of many age-related diseases including osteoarthritis [104–106]. Senescence-induced loss of replicative capacity can be induced by a multitude of stressors and signaling pathways that are associated with the aging phenotype, such as oxidative stress, telomere shortening, mitochondrial dysfunction, DNA damage and sustained cytokine activation [106–108]. As age represents the primary risk factor for OA, it is not surprising that many of these pathways are implicated in OA pathogenesis and are characteristic of the OA joint [104].

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Although there are no current studies examining the role of oxidative stress to induce senescence in the context of cartilage and OA, there is evidence that redox imbalance and oxidative stress can induce a senescent phenotype in other cells and tissues [109]. For example, deletion of SOD1 in the kidneys of mice has been shown to enhance DNA oxidative damage, upregulate markers of cellular senescence and increase production of senescence-associated secretory proteins, when compared to the kidneys of wild type mice [110], which suggests a possible connection between oxidative stress, antioxidant function and senescence [110]. Increased age-related and oxidative stress-induced telomere shortening [111, 112] DNA damage [101, 113] and mitochondrial dysfunction [6, 11] are documented in OA tissues. Since these stressors can all induce the senescent phenotype, a role for oxidative stress-induced senescence in OA appears plausible and could represent a novel and exciting avenue for OA research going forwards.

7. Redox regulation of MAP kinase activation in aging and OA

An important mechanism that contributes to the aging phenotype in cartilage and the pathogenesis of OA is an age-associated imbalance in homeostatic anabolic and catabolic signaling that leads to enhanced matrix degradation and cell death (Fig.3) [114]. The MAP kinases, which include extracellular regulated kinase (ERK), c-Jun NH2-terminal kinase (JNK) and p38, are heavily implicated in OA development and progression and are activated by a myriad of stimuli, including ROS [115]. ROS-induced phosphorylation and activation of ERK, p38 and JNK in chondrocytes has consistently been shown to initiate downstream catabolic signaling events such as release of cartilage matrix degrading enzymes [116, 117]. ROS activation of MAP kinases also contributes to inhibition of pro-anabolic signaling including IGF-1 and bone morphogenetic protein 7 (BMP7) [38, 118], downregulation of cartilage matrix synthesis [118, 119], and chondrocyte cell death [11]. In light of these observations, it has been suggested that targeting MAP kinase signaling cascades could represent a therapeutic strategy to stop or delay aging processes and the progression of age-related diseases, such as OA [101, 115].

Although the importance of ROS to stimulate MAP kinase-mediated activation of catabolic cell signaling events that lead to cartilage degradation and cell death are well documented, there remains a lack of studies examining the role of ROS in MAP kinase regulation by upstream signaling proteins. Apoptosis signal-regulating kinase-1 (ASK1) is the inaugurate protein of the MAP kinase signaling cascade. Residing upstream of JNK and p38, ASK1 can selectively activate MKK4/7-JNK and MKK3/6-p38 signaling cascades to initiate downstream catabolic signaling and regulate cell fate [120]. Activation of ASK1 has been shown to be redox dependent and is considered to be a key transducer of ROS-induced signaling [120]. The importance of ASK1 to chondrocyte survival and cartilage integrity has been examined [121]. Mouse embryonic fibroblasts (MEFs) isolated from ASK1 knockout mice and cultured in chondrogenic media to stimulate chondrocyte differentiation were treated with exogenous H_2O_2 or staurosporine to induce oxidative stress. These cells displayed a decrease in p38 and JNK signaling as well as reduced caspase-3 and PARP cleavage and lower protein levels of pro-death effector proteins BAX and BID. These findings were associated with enhanced chondrocyte survival when compared to wild type

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chondrocytes [121] suggesting that ASK1 inhibition may promote chondrocyte survival under conditions of oxidative stress through attenuating pro-death signal transduction.

Another recent study [122] by this group demonstrated that ASK1 KO mice undergoing DMM surgery to induce OA display less severe OA than WT control mice, as assessed by OARSI scores. Histologically, ASK1 KO mice displayed reduced markers of DNA strand breaks, apoptosis and MMP-13 when compared to WT mice. In addition to this post-traumatic OA mouse model, ASK1 KO mice also displayed reduced severity of age-related OA when compared to age-matched WT controls. Aged ASK1 KO mice (2 years old) exhibited increased proteoglycan content, reduced chondrocyte hypertrophy and reduced cartilage thinning when compared to WT control mice [122]. In the collagen-induced arthritis model, rats administered an ASK1 inhibitor displayed reduced inflammation and cartilage damage and decreased severity of arthritis when compared to vehicle treated controls [123]. Taken together these data suggest that targeted inhibition of ASK1 could be a therapeutic strategy to maintain chondrocyte integrity and slow cartilage degeneration in both post-traumatic and age-related OA.

8. Cysteine oxidation and redox signaling in chondrocytes

Although ROS include the highly reactive ($O_2\cdot$), hydroxyl radical and RNS, accumulating evidence suggests that H_2O_2 confers potent signaling capabilities through its ability to transiently oxidize reactive cysteine thiols on redox sensitive proteins. In physiological, reduced conditions, redox sensitive cysteine residues reside as thiolate anions ($Cys\text{-}S^-$) and are susceptible to transient oxidation by H_2O_2 to form sulfenic acid (Cys-SOH), a process termed S-sulfenylation [124]. S-sulfenylation leads to allosteric modifications within the protein, which can alter protein function. Additionally, proteins can be oxidized to form disulfide linked dimers or react with glutathione (s-glutathionylation) or other redox sensitive proteins to form mixed disulfides. Intracellular thioredoxin reductases and glutaredoxin reductases are present to reduce the sulfenic form back to the thiolate anion as part of their catalytic cycle (Fig.1). This H_2O_2-dependant dynamic and reversible oxidation process has been shown to regulate the enzymatic activity of many redox sensitive proteins including antioxidant enzymes, transcription factors, protein kinases and protein phosphatases [124].

Recent data demonstrates that chondrocytes isolated from OA cartilage display enhanced levels of basal S-sulfenyation when compared to chondrocytes isolated from non-OA cartilage [117], suggestive of an OA-associated increase in redox imbalance. When chondrocytes where treated with fragments of fibronectin (FN-f) to increase ROS levels, global protein sulfenylation was enhanced. From this pool of sulfenylated proteins, the protein tyrosine kinase Src was identified as being sulfenylated. Src sulfenylation led to enhanced Src activity and stimulated catabolic MAP kinase signaling, namely phosphorylation of JNK, which was associated with MMP-13 release, a key marker of cartilage degradation [117]. Inhibition of FN-f-induced Src sulfenylation either by pretreatment with dimedone to block FN-f-induced sulfenylation, or treatment with N-acetyl-cysteine, to decrease the level of ROS, reduced catabolic cell signaling and MMP-13 release [117]. These data suggest that ROS-induced protein S-sulfenylation may be an

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important post-translational oxidative modification capable of regulating cell signaling events in chondrocytes to promote cartilage matrix degeneration.

As reviewed in section 2.2.1, Prxs are viewed as key intermediates in H_2O_2-induced cell signaling. For example, hyperoxidation-induced inhibition of Prx function forms the foundation of the floodgate hypothesis which proposes that hyperoxidation-induced Prx inhibition allows for local increases in H_2O_2 levels and the propagation of peroxide-mediated cell signaling by sulfenylation of alternative redox sensitive proteins [125]. Alternatively, hyperoxidation-induced inhibition of Prx peroxidase activity may also reflect an adaptive mechanism to enhance Prx chaperone activities or preserve thioredoxin levels [126]. The work of Collins et al [38] demonstrates an age and OA associated increase in global Prx hyperoxidation in human cartilage sections. In chondrocyte cultures derived from human cartilage donors, aged chondrocytes also demonstrated enhanced Prx hyperoxidation both basally, and in response to H_2O_2 generated by menadione, when compared to chondrocytes derived from younger donors. Prx hyperoxidation was associated with an increase in catabolic MAP kinase phosphorylation and inhibition of the pro-survival IGF-1 pathway which ultimately led to cell death [38]. Thus, it appears that enhanced age-associated oxidative stress levels that promote Prx hyperoxidation can contribute to disturbed cell signaling events to compromise chondrocyte integrity.

9. Nuclear factor-erythroid 2-related factor (Nrf2) in aging and cartilage

In recent years, growing attention has been given to the role of the Nrf2 transcription factor in cartilage homeostasis and aging. Nrf2 is capable of regulating the basal and inducible expression of a plethora of antioxidant and detoxification enzymes, including Srx, TrxR, Prx-1, catalase, SODs, Gpx, heme-oxygenase 1 (HO-1) and NADPH:quinone oxidoreductase 1 (NQO1). Thus, maintenance of Nrf2 function is considered as an important determinant of cellular redox homeostasis [127]. Pharmacological activation of Nrf2, HO-1 [128] or genetic overexpression of Nrf2 [129] have been shown to reduce IL-1β-induced ROS generation in chondrocytes, demonstrating the importance of Nrf2 activity on the antioxidant response in cartilage.

Human OA chondrocytes have been shown to display reduced Nrf2 protein levels when compared to healthy chondrocytes in one study [130] whereas the work of others demonstrate an increase in Nrf2 protein levels in human OA cartilage [129] and human OA synovial membranes [128] when compared to non-OA donors. Additionally, Nrf2 gene expression was observed to be significantly higher in OA cartilage samples with macroscopic damage when compared to non-damaged samples of cartilage from the same OA joint [129].

In vivo mouse studies have recently highlighted the importance of Nrf2 and Nrf2-related downstream target genes in cartilage homeostasis. In an antibody-induced rheumatoid arthritis mouse model, Nrf2 knockout led to increased oxidative stress levels and enhanced markers of articular cartilage damage when compared to wild type mice [131]. Similarly, Nrf2 knockout mice have been shown to display more severe OA in both a post-traumatic model of OA (destabilization of the medial meniscus, DMM surgery)) and an inflammatory

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model of OA (monosodium iodoacetate injection, MIA) when compared to their wild type counterparts [132]. These authors also demonstrate that treatment with a histone deacetylation inhibitor (TSA) protects against DMM and MIA-induced OA through TSA-induced acetylation and activation of Nrf2. In chondrocytes, acetylation-induced Nrf2 activation led to the upregulated expression of downstream antioxidant mediators, including HO-1, which was associated with reduced matrix metalloproteinase expression [132].

HO-1 is a key downstream target of Nrf2 and protein levels of HO-1 have been shown to be significantly reduced in cartilage sections from middle aged and older mice when compared to younger mice [133]. The work of Takada et al [133] demonstrates that knockout of Bach1, a transcriptional repressor of HO-1, leads to reduced severity of age-related OA and surgical-induced OA in mice. *In vitro* studies in mouse articular chondrocytes obtained from the femoral caps of these mice demonstrated that Bach1 knockout chondrocytes displayed increased SOD2 protein levels, a key superoxide detoxifying antioxidant. Gene silencing of HO-1 reduced SOD2 levels in Bach1 knockout cells suggesting that SOD2 expression is HO-1 dependent. Additionally, silencing HO-1 led to enhanced TBHP-induced apoptosis in chondrocytes, further demonstrating a key role for HO-1 in cartilage redox homeostasis [133].

Similar reports using compounds with suggested antioxidant properties such as resveratrol [134], Licochalcone A [135], diallyl disulfide [136], Pterostilbene [137], wogonin [138], prontandin, and 6-gingerol [139] have all been shown to exert anti-inflammatory and chondroprotective properties in joint tissues through activation of Nrf2 pathways. These lines of evidence demonstrate the importance of the Nrf2 signaling pathways in maintaining redox balance and chondrocyte homeostasis during aging and in OA but the precise signaling pathways that elicit these effects remain incompletely understood. Recent data [129] suggests that Nrf2 may elicit anti-apoptotic effects via stimulation of the ERK MAP kinase pathway in response to IL-1β-induced ROS production. Further elucidating the redox signaling pathways that are altered with age, or that are governed by Nrf2 may hold therapeutic promise in cartilage aging.

10. Conclusions

There is a growing body of evidence to support a role for ROS in many of the age-related changes found in articular cartilage that alter cartilage homeostasis and contribute to the development of osteoarthritis. An increase in chondrocyte ROS levels occurs with aging. This is due at least in part to mitochondrial dysfunction resulting in increased ROS production accompanied by a decrease in the anti-oxidant capacity of chondrocytes from reduced SOD2 activity and from Prx inactivation by hyperoxidation. The resultant increase in ROS can not only cause oxidative damage to proteins, lipids and DNA resulting in increased cell death but, perhaps more importantly, can disrupt redox regulated cell signaling pathways through protein thiol oxidation (Fig.3). The latter fits with a more modern definition of oxidative stress that includes disrupted cell signaling [140]. The past failure of general, non-targeted, anti-oxidants for treating age-related conditions such as OA should not be taken as evidence that ROS are not important. There is still much to learn about the basic mechanisms responsible for age-related cartilage destruction in OA and the role of

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ROS that should lead to the development of new therapeutic strategies that will have a better chance at success.

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Highlights

- There is an age-related imbalance in chondrocyte reactive oxygen species (ROS) production and anti-oxidant capacity.
- Mitochondrial dysfunction due to aging and excessive joint loading contributes to oxidative stress in cartilage that leads to osteoarthritis.
- Excessive ROS levels in cartilage not only cause oxidative damage but alter intracellular signaling pathways to promote cartilage matrix destruction and chondrocyte death.

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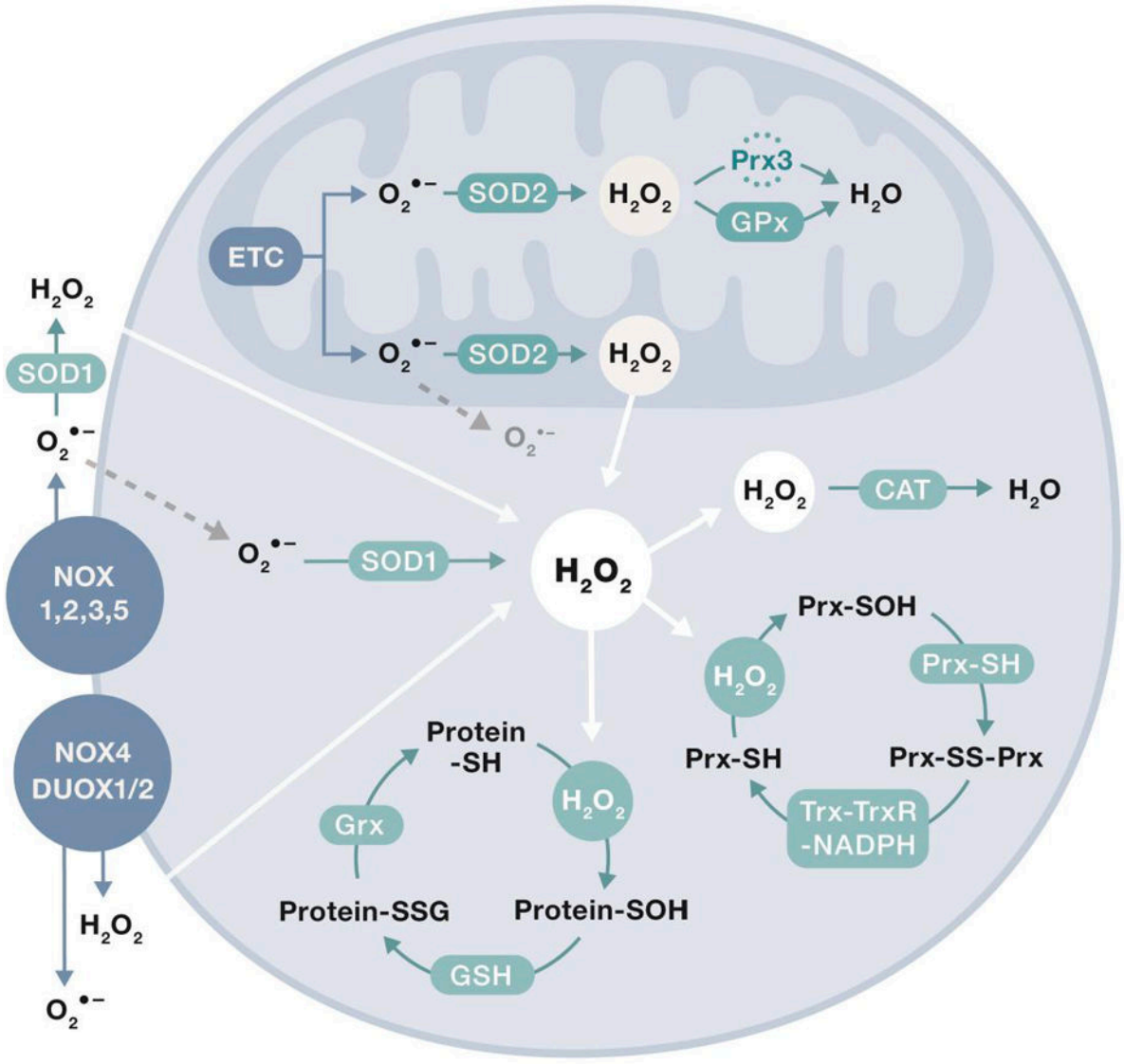

Fig 1.

Major sources and routes of metabolism for hydrogen peroxide and superoxide relevant to cartilage redox balance. Superoxide (O_2) is generated by incomplete reduction of molecular oxygen in the mitochondrial electron transport chain (ETC) and/or through NADPH oxidase enzyme (NOX) and dual oxidase (DUOX) activity. In addition to O_2, NOX4 and DUOX1/2 can also generate hydrogen peroxide H_2O_2. Often, O_2 ˙ is dismutated by superoxide dismutase (SOD) to produce H_2O_2 that is then further reduced to water by either peroxiredoxins (Prxs), catalase (CAT) or glutathione peroxidase (GPx). SOD2 and Prx3 are present in the mitochondria while SOD1 and Prx1,2,4 and 5 are cytosolic Prxs. Once oxidized, Prx dimers are reduced back to Prx monomers by thioredoxin (Trx), thioredoxin reductase (TrxR) and nicotinamide adenine dinucleotide phosphate (NADPH). H_2O_2 can also react with protein thiols (SH) to form a sulfenic acid (SOH) that can be reduced back to SH (not shown), react with a protein thiol to form a disulfide (not shown) or can react with

glutathione (GSH) to form a glutathiolated protein (-SSG) that can be reduced back to the protein thiol by glutathione reductase (Grx).

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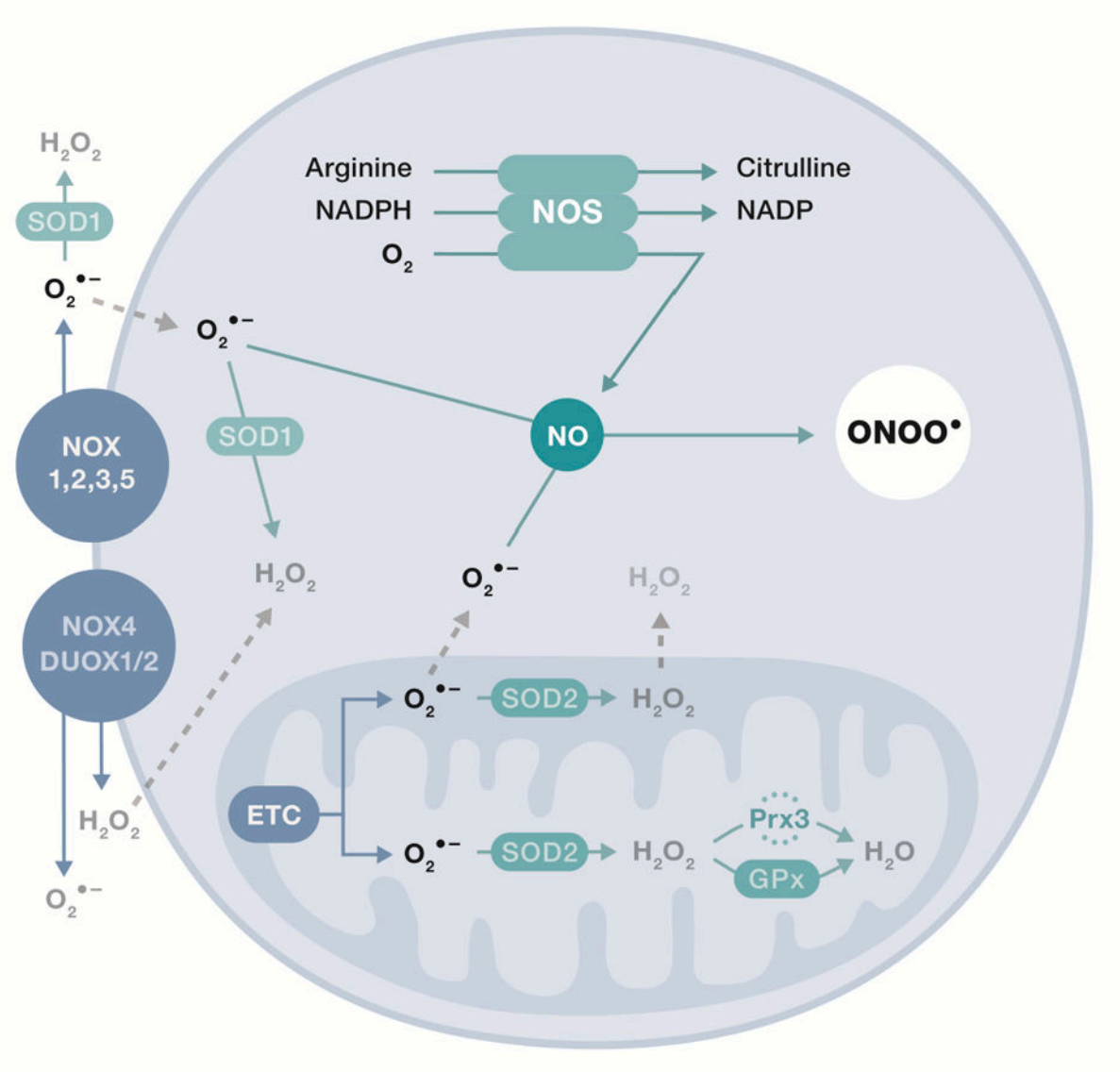

Fig 2. Nitric oxide generation and formation of peroxynitrite.

Nitric oxide synthetases (NOS) utilize oxygen (O_2), nicotinamide adenine dinucleotide phosphate (NADPH) and arginine to produce nitric oxide (NO), NADP and citrulline. NO can react with superoxide ($O_2\cdot$) (produced by sources shown in Figure 1) to form peroxynitrite (ONOO-).

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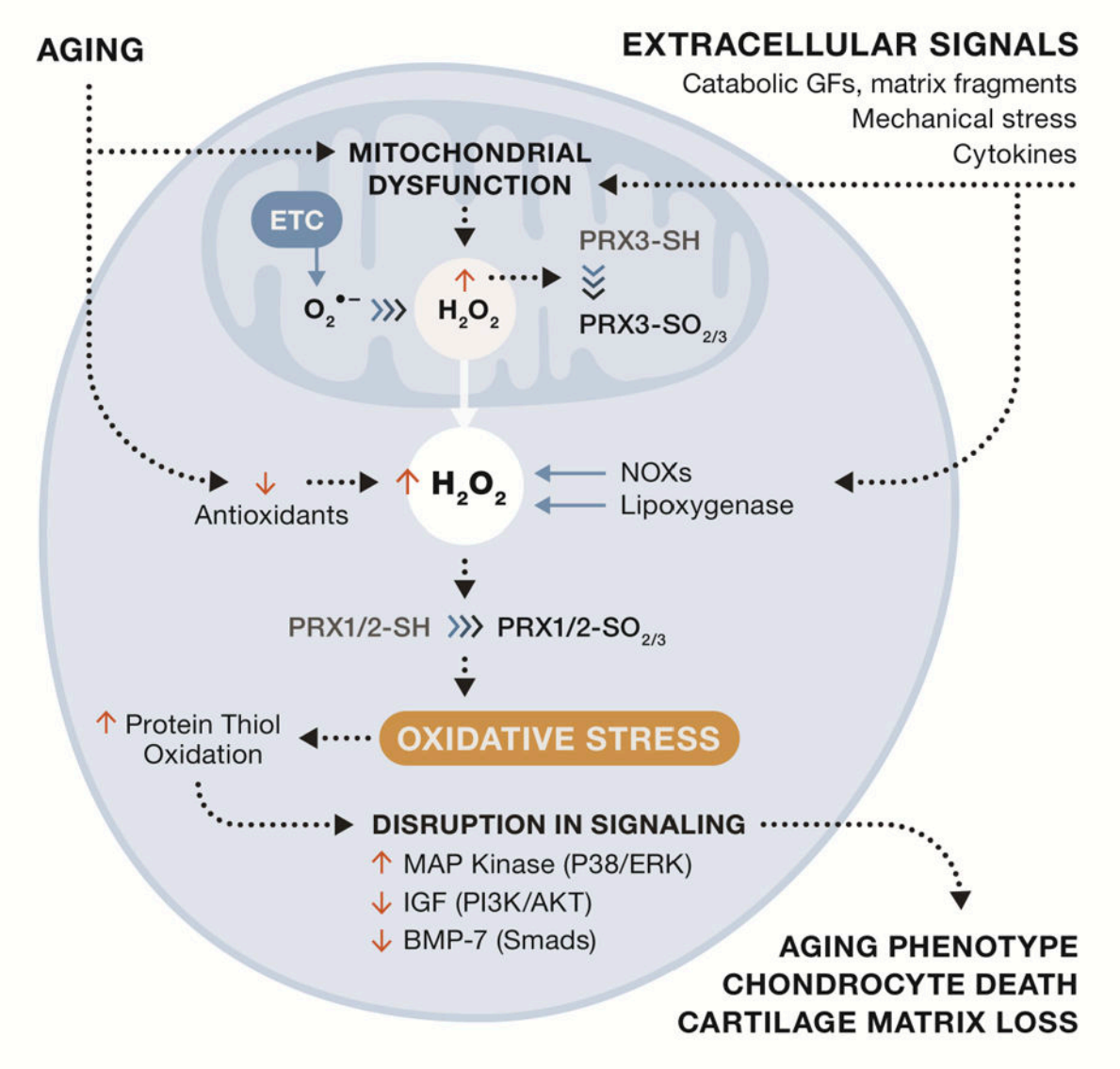

Fig 3. Mitochondrial dysfunction, oxidative stress and redox signaling in aging and osteoarthritis.

Age-associated impairment of electron transport chain function resulting in mitochondrial dysfunction coupled with reduced activity of the mitochondrial antioxidant enzymes, SOD2 and Prx3, increases oxidative stress and mitochondrial damage. In the presence of excessive levels of H_2O_2, Prx3 can be hyperoxidized to form $PrxSO_2$ or $PrxSO_3$ which results in inactivation of Prx activity. In addition, an age and OA related reduction in cytosolic antioxidant capacity alongside an increase in H_2O_2 production from various sources including NOXs and perhaps lipoxygenases, contributes to elevated levels of H_2O_2, resulting in cytosolic oxidative stress. Oxidative stress conditions lead to disturbances in homeostatic cell signal transduction, in part by oxidation (including S-sulfenylation) and hyperoxidation of redox sensitive proteins including the cytosolic Prxs 1 and 2. Disruptions in signaling identified in chondrocytes by oxidative stress include activation of pro-catabolic MAP kinase (p38 and ERK) signaling and inhibition of pro-anabolic IGF-1 and BMP7 signaling

through inhibition of PI3-kinase, Akt and Smads. This leads to an aging cell phenotype, chondrocyte death and increased cartilage matrix degradation and loss.

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