Manganese Superoxide Dismutase

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Manganese superoxide dismutase (MnSOD, also referred to as SOD2), which detoxifies superoxide, a ROS that has been shown, when its normal physiological levels are disrupted, to lead to oncogenicity and therapy resistance.

mitochondrial acetylation MnSOD ROS metabolism

1. Post-Translational Deacetylation of MnSOD by SIRT3

Sirtuins are NAD^+ dependent class III histone deacetylases that are found in various genomes from bacteria to humans [1][2][3], which deacetylate a wide range of downstream non-histone targets, including transcription factors, metabolic enzymes, and detoxification enzymes. In addition, several sirtuins have also been shown to deacetylate histones under specific conditions, including SIRT1, SIRT2, and SIRT6 [4][5][6][7]. Sirtuin proteins play important roles in metabolic and bioenergetic processes, stress resistance, cell survival, and aging [8][9]. Sirtuin genes are human and murine homologs of the *Saccharomyces* cerevisiae *Sir2* that have been shown to regulate life span and aging in model organisms, including *Saccharomyces cerevisiae, C. elegans*, and *Drosophila melanogaster* [4][10][11][12]. The sirtuin family, which constitutes the main mammalian lysine acetyl transferases, consists of seven NAD^+-dependent enzymes. These seven proteins are localized to the nucleus (*Sirt1*, 6, and 7), mitochondria (*Sirt*3, 4, and 5), and cytoplasm (*Sirt2*) and share a 275-amino-acid catalytic deacetylase domain [8].

Sirtuins, including the primary mitochondrial sirtuin, Sirt3 [2][3], appear to respond to changes in cellular and nutrient conditions, including mitochondrial stress, resulting in the activation of their deacetylase or ribosyltransferase activity, and consequently, in a post-translational modification (PTM) of downstream target proteins acetylation (Ac) levels [1][13][14][15][16]. Lysine acetylation neutralizes the positive charge on the lysine residue and can cause structural and functional changes in the protein due to changes in electrostatic potential. The researchers [17], and others [15][18][19], have shown that MnSOD is an important downstream target of SIRT3 in its role and as a mitochondrial fidelity protein [2][14][20][21]. A key aspect of sirtuins is that they require NAD+ as a co-factor, which makes them metabolic sensors and connects their enzymatic activity to the energy and redox state of cells [22][23][24]. Most mitochondrial proteins are autoacetylated due to a high acetyl-CoA concentration in mitochondria, and protein functions modified by lysine acetylation are mainly regulated by the activity of deacetylation enzymes such as SIRT3 [17][25][26][27].

Thus, an important theme that has emerged in the last several years is that SIRT3, and its most important downstream targets, direct multiple mitochondrial processes. In this regard, it appears that the non-physiological levels of MnSOD acetylation (Ac), at least in part, connect metabolic and bioenergetic balance and tumor cell growth and survival. This is based on a recently identified novel mitochondrial signaling axis centered on MnSOD-Ac, which, when dysregulated, disrupts cell metabolism, leading to aberrant ROS levels [28][29]. MnSOD is a mitochondrial matrix-localized homotetrameric antioxidant enzyme with four identical subunits, each harboring a Mn^{2+} atom [30][31]. The primary function of MnSOD is to scavenge superoxide generated from metabolic processes, including the electron transport chain. Mammalian MnSOD appears to have four lysines that have been identified as potential SIRT3 downstream targets, including K53, K89 [15][32], K68 [19][33][34], and K122 [15][16][18][19], using different methods, including site directed acetylation mutants, physical lysine acetylation followed by mass spectrometry analyses, and acetyl-lysine specific monoclonal antibodies. However, the biochemical and physiological significance of each of these lysines, and the molecular mechanism directing MnSOD enzymatic activity and mitochondrial metabolism, remains to be fully determined. The SIRT3-MnSOD-Ac axis is an active area of research in the regulation of mammalian and human cells/organs and its dysregulation appears in several human illnesses, including cancer. It now appears quite clear that the SIRT3-MnSOD-Ac axis is a mitochondrial signaling hub that regulates how cells adapt to ROS-induced metabolic stress in addition to reprograming mitochondrial metabolism, which may play an important role in late-onset diseases [25][35][36][37][38]. However, there are limited data to demonstrate the mechanism behind this idea [17][26].

2. Redox Signaling Involving MnSOD

It is proposed that there may be a mitochondrial equilibrium shift in the MnSOD tetrameric/monomeric ratio when K68 is acetylated. MnSOD catalyzes the dismutation of superoxide ($O2^{\bullet-}$) into hydrogen peroxide (H2O2); however, it has been shown to demonstrate peroxidase activity under certain conditions [33][39][40]. MnSOD is highly regulated at the transcription level by NF-kB, SP1, AP1, AP2, cytokines, and protein kinase C and is strongly correlated with cell cycle [31][41]. MnSOD is also post-translationally regulated by acetylation and phosphorylation [41][42]. Given the reversibility of the active site, tight regulation of expression and activity, and the added fact that the electron transport chain is riddled with antioxidants and only a small production of $O_2^{\bullet-}$, it has been suggested that MnSOD's second important function is as a signaling hub, as well as a superoxide dismutase [43]. This is supported by peroxidase activity [33] and the specific and selective nature in which $O_2^{\bullet-}$ and H_2O_2 oxidize targets [44][45]. H_2O_2 has a long half-life in aqueous solutions and can easily permeate mitochondria membranes; however, due to varied reduction potentials of amino acids, it has been shown that H_2O_2 selectively binds and oxidizes the active site of several phosphatases, rendering them inactive [44].

Increased MnSOD activity also leads to increased H_2O_2 signaling, inactivation of phosphatases under specific cellular and mitochondrial conditions, and an increase in phosphorylation activity of JNK/c-Jun and AMPK [44]. Overexpression of MnSOD in Jurkat cell lines led to increased phosphorylation of JNK and downstream c-Jun [44]. This pathway upregulates the expression of inflammatory cytokines and acts as a positive feedback loop for MnSOD as c-Jun is an AP-1 subunit that upregulates expression of MnSOD. JNK has been shown to prevent

tumor initiation [46] and, through the activation of this pathway, MnSOD acts as a tumor suppressor. In addition, overexpression of MnSOD, confirmed with exogenous addition of H_2O_2, led to oxidation of CaMKII-M281/282, consequently activating CaMKII [47]. This leads to phosphorylation and activation of AMPK, subsequent phosphorylation, and inactivation of acetyl-CoA carboxylase (ACC), and an increase of glycolysis [47][48]. The switch to glycolysis, i.e. the Warburg effect, is a hallmark of cancer metabolism [49][50] and, in this instance, MnSOD is acting as a tumor promoter, specifically in breast cancer, as increased MnSOD expression and glycolysis both increased as severity of breast malignancies [47]. Short-term calorie restriction can also increase H_2O_2 levels, activating AMPK [46]. The combination of results from Han, D. et al. 2020 and Han, L. et al. 2020 [51][52] show a positive feedback loop with increased SIRT3 leading to activation of the AMPK pathway and phosphorylated AMPK upregulating SIRT3 expression. [52] Phloretin was shown to mitigate the oxidative stress induced by palmitic acid and increase phosphorylation of AMPK [52]. This also correlated to increased expression of SIRT3, which was reduced when an AMPK inhibitor (dorsomorphin) was used [52].

Alternatively, inactivation of MnSOD leads to a buildup of $O_2^{\bullet-}$, which can also act as a signaling molecule that, under specific conditions, can oxidize and inactivate PTEN [53][54]. The buildup of $O_2^{\bullet-}$ due to the inactivation of SODs combines with nitric oxide to form peroxynitrite and nitrosylate PTEN, rendering it inactive [53][54][55]. This leads to increased phosphorylated (active) AKT to support cell growth. As a negative feedback loop, peroxynitrite can directly oxidize MnSOD-Tyr34, inactivating the enzyme [56]. In ER+ breast cancer, it has been reported that estrogen signaling relocates ER to the mitochondria, where it binds to MnSOD [54]. This blocks SIRT3 from binding and/or deacetylating MnSOD. The $O_2^{\bullet-}$ build-up leads to phosphorylation and activation of mTORC2, which phosphorylates and activates downstream targets AKT and PKC-α [54]. Hyper-acetylation of MnSOD can lead to over activation of AKT. Because AKT is often overactive in cancers, increasing cell survival and proliferation [57], active deacetylated MnSOD would be acting as a tumor suppressor. The totality of these results demonstrates the complexity of MnSOD's roles in cell growth and strongly implies that PTMs can significantly determine the reparative or damaging effects of this detoxification enzyme.

3. MnSOD Signaling Linked to Lineage Plasticity

MnSOD has also been linked to signaling in cancer cells through the epithelial to mesenchymal transition (EMT), also referred to as a lineage-plasticity-like phenotypic switch, leading to more aggressive phenotypes, pan anticancer resistance properties, and a metastasis permissive tumor cell phenotype [58][59][60]. There is a positive correlation in MnSOD expression and EMT score of all subtypes of breast cancer and the subsequent knockdown of MnSOD in mesenchymal cells decreases their EMT markers and morphology [60]. Conversely, it was shown that FeTPPS (a peroxynitrite scavenger) mitigates the MET (mesenchymal–epithelial transition), the reversal of EMT, and the effect of *siMnSOD*. Similarly, in small-cell lung cancer (SCLC)-derived cancer stem-like cells, overexpression of MnSOD and FOXM1 mitigated the effects of genistein, decreasing stemness/lineage-plasticity-like markers and the profound migratory/invasive activity [58], whereas knockdown of *MnSOD* and *FOXM1* enhanced the effect of genistein [58]. These studies all support the hypothesis that an increase in MnSOD can potentially lead to an increase in mitochondrial H_2O_2 concentrations, acting as a signaling molecule that increases

expression of stemness/lineage-plasticity-like markers; however, the actual MnSOD activity in cells is not addressed in these experiments.

In a quite interesting study, it was shown that the forced overexpression of MnSOD also led to an increase in acetylation of lysine 68 in breast cancer cells [34]. He et al. showed that the acetylation of MnSOD correlates with the upregulation of stemness/lineage plasticity-like biomarkers Oct4, Sox2, and Nanog through HIF2α signaling. However, He et al. also showed that the inhibition of H_2O_2 signaling with overexpression of catalase reduces the expression of HIF2α, as well as stemness biomarkers, including Oct4 and Nanog [34]. Based on these results, they postulated that MnSOD-K68-Ac leads to higher H_2O_2 levels, which adds intriguing possibilities when compared to previous reports [61][62][63]. Interestingly, the *MnSOD*^{K68Q} mutant, which is a biochemically validated acetylation mimic, also showed an increase in stemness markers [34] and provided more supporting data regarding the increased MnSOD acetylation hypothesis. A study in squamous carcinoma cells showed that the invasive cell line (A431-III) had inherently more expression of MnSOD and higher H2O2 levels than the parental cell line (A431-P) [59]. Using siRNA, the knockdown of MnSOD led to an increase in H_2O_2 and invasiveness of the cells in both the parental and metastatic cell line [59]. Treatment with antioxidants, diphenyleneiodonium, and N-acetyl-l-cysteine blocked H_2O_2 signaling and reversed the effects of the siRNA [59]. These two papers showed that either the acetylation or the downregulation of MnSOD leads to higher mitochondrial H_2O_2 concentrations, which in turn leads to a potential lineage-plasticity-like switch to cells that exhibit stemness or EMT biomarkers.

Shedding light on the seemingly opposing nature of MnSOD, as it was previously discussed when cells are manipulated to express MnSOD-K68-Ac experimentally by using the acetylation mimic *MnSOD*^{K68Q} or made in bacteria using a codon expansion method, the concentration of monomeric MnSOD also increased as a proportion and/or change in the tetrameric to monomeric ratio [33]. In addition, the monomer form of MnSOD shows peroxidase activity instead of the more established superoxide dismutase activity [33][39]. Instead of acetylation being an on/off switch, the working hypothesis is that acetylation can cause a biological change of function. This hypothesis supports the theory of MnSOD being a signaling hub. Expressing the *MnSOD*^{K68Q} acetylation mimic mutant or silencing *SIRT3* also shows a higher proportion of monomer and peroxidase activity [33]. A potential extension of these results could be that when MnSOD is overexpressed beyond its physiologically normal levels, a disproportionate increase in monomeric MnSOD occurs, which functions as a peroxidase [33]. Therefore, when it is silenced, as in Fan et al. [59], H_2O_2 concentration increases. The conditional nature of MnSOD all falls back to whether it is acetylated or not, and on the functionality of SIRT3.

4. The Cofactor of MnSOD and Peroxidase Activity, Another Layer of Regulation

MnSOD is an old, evolutionally conserved protein and is required for organisms that make ATP through respiration, primarily coming in two varieties that are categorized based on the cofactor identity. Eukaryotic MnSOD utilizes a Mn^{2+} cofactor, whereas bacteria can utilize both MnSOD and a distinct but similar FeSOD using Fe^{3+} as a cofactor. The essential structure of these enzymes is remarkably similar, especially in the active site region. In both, the active site region contains the metal cofactor ligated by three histidine residues, one aspartate residue, and one

hydroxide ion that is stabilized by a highly conserved glutamine residue. In MnSOD, the glutamine arises from the C-terminal domain (Q146 *E. coli*), but in FeSOD, the glutamine arises from the N-terminal domain (Q69 *E. coli)* [64]. Despite the similarity of the enzyme structures, the proteins behave very differently with respect to incorporation of the metal cofactor.

It has been well established that substitution of iron into MnSOD, (Fe(Mn)SOD), leads to a virtually complete loss of dismutase activity for the enzyme. It is also well understood that the structure of the enzyme is virtually unchanged upon incorporation of the "wrong" metal and the loss of activity most likely arises from a deficiency in the reduction potential of the Fe-substituted enzyme. Vance et al. suggested that Fe^{2+}(Mn)SOD is able to catalyze the reduction of $O_2^{\bullet-}$, but the Fe^{3+}(Mn)SOD generated from Fe^{2+}(Mn)SOD during the previous reaction is not able to oxidize $O_2^{\bullet-}$, and the catalytic cycle cannot be completed [65]. A similar argument can be made for Mn(Fe)SOD, which is also inactive as a dismutase. Thus, metalation is very important to these enzymes and various studies have been conducted to determine the consequences of mis-metalation. The specificity of MnSOD for the Mn cofactor is quite low. In fact, in bacteria, Fe(Mn)SOD is in 10–100 times greater abundance than MnSOD and the ratio of MnSOD/Fe(Mn)SOD is only increased under conditions of oxidative stress, such as the time when the catalytically active enzyme is needed [66]. However, within mitochondria, the enzyme is almost exclusively bound to the Mn^{2+} cofactor under normal conditions. Studies with *S. cerevisiae* have suggested that the exclusivity of MnSOD within mitochondria arises because the metalation process is coupled with the import of the unfolded polypeptide MnSOD into the mitochondria [66], and once the cofactor is incorporated into the enzyme, its removal has a high energy barrier.

Mis-metalation can be induced in *S. cerevisiae* by disruption of manganese trafficking factor (mtm1) [67]. Mtm1 plays a role in the insertion of Mn into the MnSOD enzyme and deletion of mtm1 has been shown in vivo to increase the concentration of Fe(Mn)SOD without reducing the concentration of mitochondrial manganese. Disruption of mitochondrial iron homeostasis, specifically via knockdown of *ssq1* or *grx5* [68], has also been shown to affect the MnSOD/Fe(Mn)SOD ratio in cells. Mis-metalation of MnSOD is also observed under conditions of Mn scarcity as the Fe-incorporated inactive enzyme is preferred over the apo-enzyme [69]. This is especially the case in cells where MnSOD is overexpressed [40][65][68].

Recent developments suggest that Fe(Mn)SOD has deleterious effects, not only because of its loss of dismutase function, but also because of a gained pro-oxidant/peroxidase function [70]. In early studies, this peroxidase function was attributed simply to overexpressed MnSOD, based on the oxidation of Amplex Red when MnSOD is incubated with H_2O_2 [39]. However, that interpretation has drawn some criticism [71] and several reports have shown that MnSOD is unable to oxidize $ABTS^{\cdot+}$ [70] or Amplex Red [72], whereas Fe(Mn)SOD is able to oxidize those reagents. Ganini et al. showed that, in bacteria, the peroxidase activity is highly correlated to the concentration of Fe(Mn)SOD and the dismutase activity is highly correlated to the concentration of MnSOD [40]. Further, Ganini explored the metalation of MnSOD in mammalian cells cultivated in media with different iron to manganese ratios [72]. Cells grown in Mn-deficient media were observed to have significant iron incorporation, whereas cells grown in Mn-supplemented media were mainly bound to Mn. The cultivated Fe(Mn)SOD was then shown to catalyze the oxidation of Amplex Red in the presence of H_2O_2 and supported protein radical formation. The cultivated Mn-bound

MnSOD was unable to oxidize Amplex Red and the formation of protein radicals was not observed [72]. Thus, the current evidence suggests that Fe incorporation into the MnSOD enzyme is a critical factor in the observed peroxidase activity and the role of overexpression of MnSOD is simply that it leads to a Mn deficiency in cells and makes mis-metalation more likely.

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