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Exploring the Electrostatic Repulsion Model in the Role of Sirt3 in Directing MnSOD Acetylation Status and Enzymatic Activity

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SUMMARY

Mitochondrial oxidative metabolism is the major site of ATP production as well as a significant source of reactive oxygen species (ROS) that can cause damage to critical biomolecules. It is well known that mitochondrial enzymes that scavenge ROS are targeted by stress responsive proteins to maintain the fidelity of mitochondrial function. Manganese superoxide dismutase (MnSOD) is a primary mitochondrial ROS scavenging enzyme, and in 1983 Irwin Fridovich proposed an elegant chemical mechanism/model whereby acetylation directs MnSOD enzymatic activity. He christened it the “electrostatic repulsion model”. However, the biochemical and genetic mechanism(s) determining how acetylation directs activity and the reasons behind the evolutionarily conserved need for several layers of transcriptional and post-translational MnSOD regulation remain unknown. In this regard, we and others have shown that MnSOD is regulated, at least in part, by the deacetylation of specific conserved lysines in a reaction catalyzed by the mitochondrial sirtuin, Sirt3. We speculate that the regulation of MnSOD activity by lysine acetylation via an electrostatic repulsion mechanism is a conserved and critical aspect of MnSOD regulation necessary to maintain mitochondrial homeostasis.

Keywords

Electrostatic Repulsion Model; MnSOD; Sirt3; Sirtuins; Metabolism; Mitochondria; ROS; Acetylation; Acetylome; Metabolic homeostasis

Acetylation in the regulation of mitochondrial proteins

Eukaryotic cells contain post-translational adaptive signaling pathways for responding to oxidative stress that induce the enzymatic activity of ROS scavenging proteins to maintain metabolic homeostatic poise. These signaling pathways permit the cell to exploit and adapt

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to environmental conditions, thereby minimizing the potentially deleterious effects of oxidative metabolism. Over the last few years, several proteomic studies have suggested that the cellular acetylome is roughly 2/3 the size of the kinome, suggesting that protein acetylation may play a key role in the regulation of signaling cascades [1–3]. Acetylation of lysine residues neutralizes positive charges on proteins, subsequently altering their 3-dimensional structure as well as changing enzymatic function [4, 5]. In this regard, lysine acetylation has emerged as a critical post-translational modification employed to modify mitochondrial proteins as well as regulate protein catalytic activity [3, 6]. The idea that the mitochondrial acetylome directs cellular metabolism is based on several proteomic surveys identifying a high number of acetylated proteins in the mitochondria that appear to direct metabolism [1, 3]. These studies clearly suggested that the acetylome, via changes in lysine acetylation, regulates a wide spectrum of metabolic pathways employed to direct energy production [1, 3, 7] in a carbon source-dependent fashion [8].

A key observation regarding mitochondrial biology is that mice placed on a caloric restriction (CR) diet display a very significant change in both global and mitochondrial protein acetylation [9, 10]. For example, large-scale mass spectrometry screening pre- and post-CR identified well over 100 reversibly acetylated lysines in 72 mitochondrial proteins from a wide variety of metabolic pathways [11]. Interestingly, CR or other forms of nutrient stress have been investigated for many years and have been shown to prevent or reverse age-related changes in multiple murine phenotypes, including insulin resistance and neurodegenerative diseases, and to decrease the incidence of carcinogenesis in tumor permissive mice. Thus, over the last few years there has been mounting evidence that various forms of nutrient stress, such as exhaustive exercise, fasting, or CR, can profoundly alter mitochondrial physiology and pathophysiology associated with aging as well as age-related illnesses [12]. Based on these results, it seems reasonable to suggest that acetylation of mitochondrial proteins may play a role in maintaining and regulating mitochondrial metabolism and function.

The importance of metabolic homeostasis

For many years it has been known that the maintenance of metabolic homeostasis in mammalian cells requires a series of biochemical oxidation/reduction (redox) reactions involving protein catalysts [13, 14]. Mammalian cellular processes primarily derive energy from the tightly controlled regulation of oxidative metabolism, which biochemically oxidizes substrates (i.e. carbohydrates, fats, and amino acids) [15, 16]. The oxidation of substrates is used to obtain reducing equivalents (electrons) necessary for mitochondrial electron transport-mediated oxidative phosphorylation to produce ATP, with O_2 acting as the terminal electron acceptor [17–19]. Furthermore, reducing equivalents are also involved in many cellular processes including gene replication and transcription, protein synthesis as well as maintaining the cellular reducing environment [14, 20, 21]. However, oxidative metabolism is also capable of generating ROS [e.g., superoxide ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($HO\bullet$)] as byproducts [22, 23] that can act as both signaling molecules and damaging agents. Through a series of biochemical oxidation/reduction (redox) reaction processes, $O_2^{\bullet-}$ is dismutated to hydrogen peroxide H_2O_2 either spontaneously or enzymatically in the presence of superoxide dismutase enzymes [i.e., copper zinc superoxide dismutase (CuZnSOD), manganese superoxide dismutase (MnSOD), extracellular superoxide dismutase (EcSOD)] [24–26].

Steady-state levels of ROS are a function of both production via aerobic respiration and removal by antioxidant scavenging enzymes [22, 27]. Under normal circumstances, it is estimated that 1% or less of total O_2 consumption results in $O_2^{\bullet-}$ and H_2O_2 formation through mitochondrial electron transport chains [28, 29]. This low level of ROS is essential

for many normal cellular processes including cell signaling, cell adhesion, cellular immune response, apoptosis and cell survival [30–33]. In addition, antioxidant capacity can shift metabolism away from pathways that produce ROS as well as prevent ROS reactions with critical biomolecules. However, under certain circumstances like exposure to exogenous stress or the aging process, metabolic prooxidant production can exceed antioxidant capacity, leading to oxidative stress [22, 34]. The excess ROS can then react with a broad range of biomolecules including lipids, proteins and DNA to form other radicals or cytotoxic byproducts. For instance, lipid peroxidation can lead to the oxidative degradation of lipids to form reactive and cytotoxic products [31]. Since this process proceeds by a free radical chain reaction mechanism, lipid peroxidation not only affects polyunsaturated fatty acids, but it could also amplify the number of free radicals as well as produce diffusible cytotoxic byproducts [15]. It is widely thought that maintaining the balance of metabolic homeostasis is critical to cell fate and that increased ROS production by abnormal redox metabolism leads to disrupted regulation of gene expression and aberrant protein activities that could contribute to cell injury, mutagenesis, senescence, teratogenesis, carcinogenesis, and cell death [35–37].

MnSOD in the maintenance of cellular metabolic homeostasis

The proper regulation of mitochondrial function as well as maintenance of mitochondrial oxidative metabolism is critical to minimize the accumulation of potentially damaging ROS [38]. In addition, the mitochondria represent the primary site of superoxide production through the process of respiration via the electron transport chain [39, 40]. While low levels of superoxide and/or other ROS are easily tolerated by the cell, abnormally high levels of ROS from any number of possible sources induce oxidative stress and can damage cells [25]. As such, the mitochondria contain specific processes to scavenge and remove ROS in order to maintain homeostasis.

In humans there are three forms of SOD: cytosolic CuZnSOD, mitochondrial MnSOD, and EcSOD [37]. Among them, MnSOD is the major enzymatic superoxide scavenger inside the mitochondrial matrix and is considered one of the most critical ROS scavenging/detoxification enzymes in the cell, one that is absolutely necessary for an organism to survive and produce ATP in an oxygen-rich environment [41, 42]. Human MnSOD is a tetrameric enzyme complex that is made up of four identical subunits each harboring a Mn^{3+} atom (Fig. 1A) that converts two $O_2^{\bullet-}$ molecules to one O_2 and one H_2O_2 through a ping-pong catalytic mechanism characterized by redox transitions of Mn^{3+} to Mn^{2+} and back to Mn^{3+} [24, 43, 44]. Since the production of $O_2^{\bullet-}$ is a function of the rate of one-electron reductions of O_2 within the mitochondria, it seems reasonable to propose that the mitochondria would contain watchdog or sensing proteins to regulate oxidative metabolic processes, including the activity of detoxification enzymes such as MnSOD, to maintain metabolic homeostasis as well as prevent cellular damage during oxidative metabolism.

Sirt3 deacetylates and regulates MnSOD enzymatic activity

As suggested above, lysine acetylation has recently emerged as an important post-translational modification employed to regulate mitochondrial proteins [45–47]. Thus, lysine acetylation, regulated by either acetyl transferases or deacetylases, may play an important role in metabolic homeostasis by coordinating mitochondrial oxidative metabolism and ROS levels to match ATP production with intracellular energy requirements. In this regard, a new family of mammalian lysine acetyl transferases, referred to as sirtuins, were discovered early in the last decade consisting of seven NAD⁺-dependent proteins sharing a common 275-amino acid catalytic domain [48]. These sirtuins are localized to the nucleus (Sirt1, 6, and 7), mitochondria (Sirt3, 4, and 5), and cytoplasm (Sirt2) and require NAD⁺ as a cofactor. It

now appears clear that the mammalian sirtuin protein family connects its enzymatic deacetylation activity to the regulation of cellular and mitochondrial metabolism as well as provides a mechanistic link between the sirtuin activities, energy production, and scavenging pathways that prevent oxidative stress [49, 50].

Sirt3 is the primary mitochondrial deacetylase [51] and directs biological functions involved in mitochondrial energy production [52] as well as to limiting the accumulation of mitochondrial ROS [53, 54]. For example, Sirt3 appears to regulate energy producing mitochondrial proteins including acetyl-coenzyme A synthetase, long-chain acyl-coenzyme A dehydrogenase, and 3-hydroxy-3-methylglutaryl coenzyme A synthase 2 and enhances their enzymatic activity in responding to nutrient distress [52, 55–57]. Thus, it seems logical to propose that Sirt3 functions as a sensing or fidelity protein which can regulate downstream targets through post-translational modifications involving protein acetylation to modify cellular metabolism and redox balance in response to nutritional demands.

In the last two years, three seminal papers have been published that demonstrated that deacetylation of MnSOD by Sirt3 directs MnSOD enzymatic activity and identified the target lysines [58–60]. These studies, as well as others [61, 62], also showed that CR, fasting, and other forms of oxidative stress induce both Sirt3 deacetylation activity and MnSOD activity. In addition, it is now well established that overexpression of Sirt3 decreases both intracellular $O_2^{\bullet-}$ and total ROS levels, suggesting that Sirt3 regulates both energy production and mitochondrial ROS scavenging pathways. These results raise several significant questions regarding the regulation of MnSOD by changes in lysine acetylation that include: (1) what is the mechanism whereby a change in lysine acetylation alters the enzymatic activity? and (2) why do changes in nutrient status and/or CR increase MnSOD activity?

Mitochondrial superoxide and other ROS as second messenger signaling molecules

While producing ATP and maintaining energy homeostasis are essential mitochondrial processes, it has been noted that the mitochondria may also serve as primary cellular sources of endogenous oxidants. In this regard, ROS are continuously produced by oxidative phosphorylation pathways consisting of five multisubunit enzyme complexes embedded within the mitochondrial inner membrane. Sirt3 deacetylates mitochondrial protein targets relevant to increasing tricarboxylic acid cycle activity (IDH, GDH, acyl-CoA synthase) and electron transport chain activity (Complexes I–III), which increases electron fluxes leading to superoxide formation in mitochondria. In addition, the levels of mitochondrial ROS are also determined by scavenging enzymes and thus, a balance is established between production and removal by a series of interconnected pathways [24, 63]. As such, it seems logical to propose that Sirt3 deacetylation of MnSOD could increase the capacity to convert superoxide to H_2O_2 , potentially protecting cells from the effects of increased fluxes of superoxide as well as signaling the mitochondrial metabolic shift to cytosolic signaling pathways necessary for readjusting gene expression to the new metabolic steady-state [22, 64, 65]. In this regard, many current studies propose that there is a physiological range in which ROS can function as critical signaling molecules [23, 66]. However, this raises the question as to how changes in mitochondrial $O_2^{\bullet-}$ and ROS levels might alter metabolic processes in other cellular compartments and further control cell fate, i.e., the choice between cell proliferation and cell death.

A fundamental paradigm in biology is that different parts of the cell, including organelles, communicate with each other via signaling pathways to maintain cellular homeostasis; however, while it has been shown that signals can be sent into the mitochondria, it is not

clear if the mitochondria can communicate reciprocally with other parts of the cell [46, 67]. For mitochondrial free radical production to serve as signaling molecules it seems reasonable to propose that there may be a functional connection or cross talk between the mitochondria, cytoplasm, and nucleus when responding to specific cellular conditions, including those that would activate Sirt3. In the last few years several groups have proposed a model in which aberrant levels of ROS induce depolarization of mitochondrial membrane potential, which is then propagated throughout the cell via a mechanism referred to as ROS-induced ROS release (RIRR) [68–70]. Specifically, it has been proposed that RIRR may be a means whereby mitochondria communicate with each other, as well as other parts of the cell, via the diffusion and regeneration of small ROS messenger molecules [67]. The compartmentalization of ROS production within a cell is critical to its signaling function and is facilitated by intracellular localization of specific scavengers.

Based on these ideas, as well as reports demonstrating that MnSOD activity is directed by conditions that induce specific forms of cell stress, it is proposed that changes in MnSOD lysine acetylation may play a critical role in how the mitochondria communicate with other parts of the cell [54, 58, 71, 72]. This idea is consistent with signaling pathways observations in the cytoplasm and nucleus; however, the mitochondria appear to use changes in ROS as second message signaling [73–77]. In some ways this seems logical since the mitochondria make $O_2^{\bullet-}$ and ROS as a consequence of respiration and as such, ROS would make an ideal second messenger to communicate with other cellular compartments.

In support of this hypothesis, it has been previously shown that mitochondrially generated ROS can serve as signaling or communicating molecules between different cellular organelles, where the steady-state levels of mitochondrial superoxide and H_2O_2 could serve as a sensing mechanism for controlling key cellular processes such as proliferation and apoptosis. For example, Venkataramn et al. showed in PC-3 cells that overexpression of MnSOD resulted in a delay of the G1-S phase transition, a delay mediated, at least in part, by modulation of the redox status of the cell through the increased levels of H_2O_2 [78]. In addition, Karawajew et al. demonstrated that mitochondrial ROS served as a secondary messenger to guide p53 translocation to the mitochondria, leading to the activation of apoptosis and p53 targeted gene expression [73]. They also showed that cells treated with oligomycin, which inhibits ATP synthase, prevented stress-induced mitochondrial accumulation of p53 protein and abrogated p53-dependent apoptosis by reducing mitochondrial ROS levels [73]. Similar experiments with exposure to FCCP (a mitochondrial electron uncoupling agent) also showed decreased stress-induced p53 mitochondrial translocation and superoxide production [74]. These results, as well as many others, suggest that increased mitochondrial $O_2^{\bullet-}$ may function as a molecular messenger and support the hypothesis that the alteration of mitochondrial ROS production, via changes in MnSOD enzymatic activity, represents a potential mechanism for intra-compartmental cellular communication [22, 23, 27, 28].

These results also raise an intriguing question: what is the physiological need for which CR and/or fasting results in MnSOD deacetylation and increased MnSOD catalytic activity? One possible explanation may be that nutrient distress requires significant changes in how the mitochondria should generate ATP. For example, we would hypothesize that under nutrient deprivation, mitochondria would shift energy production to mitochondrial oxidative phosphorylation since this is the most efficient way to make ATP from available carbon sources. This shift might be expected to necessitate the activation of MnSOD to protect the cell from $O_2^{\bullet-}$ as well as shifting the balance of intracellular oxidants towards H_2O_2 , which could act as a second messenger to the rest of the cell to maximize mitochondrial energy production in an environment where there is a relative shortage of potential carbon sources.

An electrostatic mechanism directing MnSOD activity

Roughly thirty years ago, in 1983, Fridovich et al. proposed a new mechanism for directing MnSOD enzymatic activity through changes in acetylation/deacetylation, which they coined “electrostatic facilitation” [77]. Under this hypothesis, $O_2^{\bullet-}$ had to travel into the center of the MnSOD tetrameric complex through an electrostatic funnel where the positive charges of lysine residues drew superoxide toward the active site (Fig. 1A). Using an elegant set of chemical and biochemical studies, it was proposed that a cluster of positively charged amino acids, possibly lysines, could form a cationic region to attract the superoxide and thus increase the activity of MnSOD [77]. At that time it was not possible to perform high-resolution MnSOD structure analysis or construct lysine site-directed mutants and as such, identifying the reversibly acetylated lysine responsible for this activity-abetting effect was not possible. However, his work clearly suggested that the lysine involved would be on the outside of the protein near the entrance to the MnSOD inner catalytic core.

In this regard, a recent publication [58] has identified a specific lysine located at amino acid position 122, near the entrance to the MnSOD inner catalytic core, that appears to regulate MnSOD activity via reversible acetylation. The data presented in this paper provide significant experimental data to validate the electrostatic facilitation model proposed by Dr. Fridovich. Further 3-D structure and sequence analysis showed that lysine 122, which is conserved in multiple species, is located close to the entrance to the catalytic core and ideally oriented to provide superoxide anion attraction (Fig. 1B). In addition, this report showed that when lysine 122 was mutated to an arginine (positive charge mimicking a deacetylated state, MnSOD^{K122-R}) MnSOD activity was increased, intracellular ROS were decreased, and stress-induced genomic instability was prevented. In contrast, when lysine 122 was changed to a glutamine (neutral charge mimicking an acetylated state, MnSOD^{K122-Q}) MnSOD activity was decreased. As such, we propose that when lysine 122 is deacetylated by Sirt3 in MnSOD, the electrostatic funnel remains positively charged and thus superoxide is more readily delivered to the active site and for conversion to hydrogen peroxide. However, when lysine 122 is acetylated, the electrostatic funnel shows a neutral to negative charge which repels superoxide and thus decreases the possibility of superoxide entering the active site. Thus, these results strongly suggested that lysine acetylation status directs MnSOD enzymatic activity and ROS levels through an electrostatic facilitation mechanism as elegantly proposed over thirty years ago.

Conclusions

Mitochondrial ROS play a significant role in many cellular physiological processes, and the key regulator of mitochondrial ROS inside the inner mitochondrial membrane is MnSOD. It now appears clear that Sirt3 is a mitochondrial sensing or watchdog protein that directs the activity of a series of critical downstream mitochondrial proteins, including MnSOD superoxide detoxification activity. In this review we propose a model for regulation that was initially presented thirty years ago, coined “electrostatic facilitation” at that time. However, it seems clear that protein acetylation and deacetylation direct enzymatic activity by altering protein dynamics as well, similar to that observed for other types of post-translational modifications [79]. In this regard, deacetylation of HMGCS2 at lysines 310 and 447 has been shown, via molecular dynamics simulations, to significantly affect protein conformation, dynamics, and electrostatics near the active catalytic site while minimally affecting the remainder of the protein [80]. MnSOD contains potentially reversibly acetylated lysines at locations similar to those in HMGCS2, suggesting that conformation and dynamics may play an equally important role in directing MnSOD activity.

Finally, we also speculate that the regulation of MnSOD activity via changes in lysine acetylation may play a role, at least in part, to transiently alter the steady-state levels and/or ratio of $O_2^{\bullet-}$ to H_2O_2 that may function as second messengers whereby mitochondrial oxidative metabolism can communicate redox signals to other cellular compartments. Since superoxide primarily mediates one electron signaling through metal ions associated with protein activity (i.e., aconitase, Fe-S proteins associated with electron transport chains, and prolyl hydroxylase) and hydrogen peroxide primarily mediates two electron signaling through redox sensitive thiol residues on critical signaling proteins (i.e., transcription factors, phosphatases, and kinases), changes in the activity of MnSOD, mediated by Sirt3 deacetylation (linked to $NAD^+/NADH$ ratios), may shift steady-state levels of $O_2^{\bullet-}$ to H_2O_2 to rapidly transmit metabolic redox signals to proteins critical for both normal cellular transitions as well as responses to oxidative stress [22, 23]. In this regard Sirt3 may act as a master regulator linking oxidative metabolism to signal transduction and gene expression via the ability to alter redox signaling through changes in MnSOD activity. While our data suggest these conclusions, much more work will be required to rigorously validate these hypotheses.

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Abbreviations

ROS	Reactive oxygen species
CR	Caloric restriction
MnSOD	Manganese superoxide dismutase
CuZnSOD	Copper zinc superoxide dismutase
EcSOD	Extracellular superoxide dismutase
$O_2^{\bullet-}$	Superoxide
H_2O_2	Hydrogen peroxide
$HO\bullet$	Hydroxyl radical
Sirt1-7	Sirtuin-1-7
RIRR	ROS-induced ROS release

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Highlights

- MnSOD enzymatic activity is directed by lysine acetylation status
- Sirt3 deacetylates MnSOD and alters enzymatic activity
- Electrostatic Repulsion Model connects Sirt3 and MnSOD enzymatic activity

Figure 1. Proposed model describing the electrostatic mechanism for how lysine acetylation by Sirt3 affects MnSOD activity

In this model decrease or loss of Sirt3 deacetylation activity results in an alteration of the charge distribution within the electrostatic funnel and further contributes to the decreased MnSOD activity. **(A)** Sirt3 deacetylation regulates the electric charge at the opening of the electrostatic funnel. Decrease or loss of Sirt3 activity (left) results in hyperacetylation of MnSOD (lysine 122) and induces neutral to negative charge within the electrostatic funnel, thus repelling the O₂^{•-} from the active site of MnSOD and preventing its conversion into H₂O₂. In contrast, functional Sirt3 (right) removes the acetyl group of lysine 122 and creates a positive environment which attracts more O₂^{•-} into the active site, facilitating MnSOD enzymatic output. **(B)** An electrostatic view of the funnel entrance of MnSOD. Hyperacetylated MnSOD results in a overall neutral to negative charge at the funnel entrance (left), while deacetylated MnSOD results in an overall positive charge at the funnel (right).