Perspective

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Exercise-induced fumarate accumulation: a potential mediator of mitochondrial biogenesis in mammalian skeletal muscle

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Abstract

Endurance exercise is known to induce mitochondrial biogenesis in mammalian skeletal muscles. However, the exact mechanism via which this exercise-induced mitochondria biogenesis occurs remains unclear. Extraneous physical activity induces several adaptive metabolic changes in the skeletal muscle including the activation purine nucleotide cycle (PNC) and utilization of amino acid as fuel sources. These metabolic adaptations are likely to cause accumulation of intra-muscular fumarate that might be a key mediator of exercise-induced mitochondrial biogenesis. This is because high intracellular levels of fumarate cause irreversible succination of Keap1, which leads to activation of nuclear factor erythroid-derived 2-like 2 (NFE2L2, Nrf2) signalling. Activation of Nrf2 signalling in turn is known to play a role in contraction-induced mitochondria biogenesis. Accordingly, it is possible that therapeutic strategies that enhance skeletal muscle fumarate levels might potentially enhance muscle mitochondrial biogenesis, content and overall physical performance.

Keywords: Exercise, Fumarate, Mitochondrial biogenesis.

INTRODUCTION

Chronic skeletal muscle contractile activity, such as during endurance training, often induces mitochondrial biogenesis, leading to increased muscle mitochondrial content and functions [1-6]. In contrast, diminished contractile activity impairs mitochondrial biogenesis with detrimental health consequences including muscle atrophy [7-12]. However, the exact mechanisms via which changes in contractile activity level modulate skeletal muscle mitochondrial biogenesis remains to be fully elucidated.

Several studies have demonstrated that increase in muscle contractile activity activates nuclear factor erythroid-derived 2-like 2 (NFE2L2, Nrf2) signalling [13-17]. Moreover, knockout of Nrf2 in mice has been shown to attenuate endurance exercise-induced mitochondrial biogenesis in skeletal muscles [15]. These findings indicate that Nrf2 signalling plays a key role in contraction-induced mitochondrial biogenesis. However, the exact mechanism via which muscle contraction activates and regulates this Nrf2-dependent mitochondrial biogenesis is still unclear.

Under basal cellular conditions, Nrf2 signalling is repressed by Keap1, which facilitates continuous ubiquitylation of Nrf2 by cullin 3-RING box protein (Cul3-RBX1) E3 ligase and targeting for proteosomal degradation. Upon increase in intracellular oxidants or electrophiles, critical cysteine residues in Keap1 protein are covalently modified, thereby abrogating its ability to target Nrf2 for proteosomal degradation [18-20]. This in turn promotes the accumulation of Nrf2, translocation to the nucleus and subsequent activation of antioxidant response element (ARE)-driven transcription [18-20].

Notably, skeletal muscle contraction leads to formation of reactive oxygen species (ROS) [21-24], nitric oxide (NO) and reactive nitrogen species (RNS) [25]. Therefore, skeletal muscle contraction can activate mitochondrial biogenesis via ROS- and NO/RNS-mediated activation of Nrf2 signaling. Indeed, a study in mice demonstrated that exercise induces activation of Nrf2 signaling [15]. However, it is important to note that activation of Nrf2 signalling induces various intracellular antioxidant systems, which are known to effectively limit the levels of ROS and RNS [15, 26]. This would inevitably diminish the role and effectiveness of contraction-induced ROS and NO/RNS in the activation of Nrf2-dependent mitochondrial biogenesis

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over time. It is therefore likely that other more robust contraction-related mediators are involved in the induction of adaptations such as mitochondrial biogenesis that require several weeks of sustained activation of Nrf2. Herein, fumarate-mediated activation of Nrf2 is considered as one of the potential mechanisms, via which chronic exercise promotes mitochondrial biogenesis in skeletal muscles.

Skeletal muscle contraction increases fumarate levels

Several studies have reported fumarate as among the intermediate metabolites whose concentrations increases the most upon stimulation of muscle contraction. Aragon and Lowenstein [27], for example, stimulated rat hind limb contraction *insitu* using electric pulses and measured tricarboxylic acid cycle intermediates (TCAI) after 10 min. They demonstrated up to fourfold increase in fumarate and malate concentrations, which they attributed mainly to increased activation of the purine nucleotide cycle (PNC). Gibala et al[28] examined changes in human skeletal muscle TCAI levels during a short (5 min) dynamic knee extensor exercise. They observed two-three times increase in succinate, fumarate and malate concentrations, which was likely due to initial increase in the flux through the AAT reaction. The same group futher examined TCAI changes following a regime that consisted of moderate 10 min leg extensor exercise followed immediately by intense exercise until exhaustion. They observed a two-fold increase in fumarate levels after the 10 min of moderate exercise, which was further increased by the intense exercise phase to reach concentration that is four-fold of the rest value at the time of exhaustion [29]. In a more prolonged endurance-type cycling exercise, the intramuscular fumarate was shown to increase rapidly to a peak concentration that is six times higher than at rest before falling and remaining at three-fold higher concentrations until exhaustion time [30]. More recently, measurement done in human plasma also revealed two-fourfold increase in fumarate, succinate and malate levels following acute exercise or marathon running [31]. This closely mirrors the observations made previously in muscle homogenates [27-30]. Collectively, these observations indicate that contraction induces a substantial increase in fumarate levels across all the muscle compartments including its extracellular space.

More importantly, studies indicate that this contraction-induced increase in the levels of fumarate and other TCAI persist during prolonged endurance training. Howarth et al[32], for example, investigated exercise-induced changes in the concentration of malate, fumarate, citrate and isocitrate before and after seven weeks of endurance training. After seven weeks of training, the intramuscular fumarate was shown to increase by more than 5 times of the resting value, indicating that fumarate increase can act as robust contraction-related signal. Notable though, is that the seven-week training attenuated the overall increase of TCAI that usually occur during the initial stage of exercise, which was consistent with reduction in the net flux through the AAT reaction that occur at the start of exercise [32].

Besides, the PNC, increased utilization of amino acids as carbon sources for the tricarboxylic acid cycle (TCA) reactions might also contribute towards build-up of fumarate during muscle contraction. This is because during the initial stages of exercise for example, the activity of alanine amino transferase (AAT) is markedly enhanced, thereby promoting increased entry of glutamate into the TCA cycle as α-ketoglutarate [33]. In the later stages of prolonged strenuous exercise, the branched chain amino acids (isoleucine, leucine and valine) are increasingly oxidised and channelled into the TCA cycle mainly at the level of succinyl CoA [33-35], causing rapid expansion of downstream TCA intermediates (TCAI) including fumarate.

Fumarate activates Nrf2 signalling via Keap1 succination

Fumarate is an electrophile, which when present at high concentration reacts with cysteine residues thiol (–SH) groups to form S-(2-succinyl) cysteine (2SC) adducts, a process termed succination [36, 37]. This succination reaction is irreversible, causing long-term structural and functional modification of the affected proteins. Because of this irreversibility, succination is considered a post-translational modification mechanism that is involved in the long-term regulation or dysregulation of cellular functions, including metabolism [36-40]. Keap1 has reactive cysteine residues in its structure and is thus a target for succination. This has been clearly demonstrated in cells that accumulate high fumarate due to fumarate hydratase defects. In such cells, critical Keap1 cysteine residues are irreversibly succinated, causing conformational modification in Keap1 protein structure, with resultant activation of Nrf2 signalling [38, 41]. Besides succination of Keap1, fumarate is also known to competitively inhibit α-ketoglutarate (α-KG)-dependent DNA demethylases, leading to global DNA hypermethylation [42-45]. Increased methylation of Keap1 gene promoter inhibits its expression [46-49]. Therefore, high cellular fumarate might not only inhibit Keap1 at the post-translational level but might also inhibit Keap1 expression at the mRNA level. This can reduce the turnover of the irreversibly succinated Keap1 protein molecules. The consequence of which is prolonged and robust activation of Nrf2 signalling.

Moreover, fumarate also forms succinate adducts with glutathione (GSH), thereby diminishing intracellular GSH and NADPH levels, with resultant increase in cellular oxidative stress [50, 51]. This could facilitate continuous activation of Nrf2-dependent responses by generated ROS or RNS even in the face of increasing Nrf2-mediated induction of GSH/dependent antioxidant systems. This could lead to synergistic or additive activation of Nrf2 during chronic contractile activity, where increased generation of both fumarate and ROS is likely to occur. In summary, therefore, fumarate can robustly activate Nrf2 signalling via multiple mechanisms, including Keap1 succination, decreased expression of Keap1 gene and depletion of GSH.

Activation of Nrf2 signaling drives NRF1-mediated mitochondrial biogenesis

Mitochondrial biogenesis is a highly complex process that requires regulated transcription and translation of several mitochondrial DNA (mtDNA) and nuclear encoded mitochondrial genes, importation of several peptides from the cytosol and replication of mtDNA [52, 53]. Among the key factors regulating these processes that are necessary for mitochondrial biogenesis is nuclear respiratory factor 1 (NRF1). NRF1 promotes the expression of various nuclear-encoded respiratory chain subunits and mitochondrial RNA processing (MRP) RNA [54]. NRF1 also activates the expression of 5-aminolevulinate synthase, a key rate-limiting enzyme in the synthesis of heme [55] as well as that of mitochondrial transcription factor A (TFAM), a major regulator of mtDNA replication and transcription [56, 57]. In addition, NRF1 activates the expression of mitochondrial transcription specificity factors (TFB1M and TFB2M) that enhances mtDNA transcription in the presence of TFAM and mitochondrial RNA polymerase [58]. Therefore, increased NRF1 expression enhances mitochondrial biogenesis. Importantly, NRF1 gene promoter has been shown to have four antioxidant response elements (ARE), through which Nrf2 can drive its expression with resultant induction of adaptive mitochondrial biogenesis [59].

Therefore, a fumarate-Nrf2-NRF1 signalling axis via which exercise can promote mitochondrial biogenesis can be proposed. In this axis, increased skeletal muscle contraction leads to elevated intramascular levels of fumarate. This elevated fumarate might in turn inhibit muscle Keap1 via succination and probably epigenetic mechanisms, thereby enhancing Nrf2 protein stabilization, translocation into the nucleus and transactivation of ARE-containing target genes including NRF1. ARE-driven expression of NRF1 would in turn induce mitochondrial biogenesis by promoting the expressing of various nuclear DNA and

mtDNA encoded genes, as wells as replication of mtDNA, altogether leading to enhanced mitochondrial biogenesis.

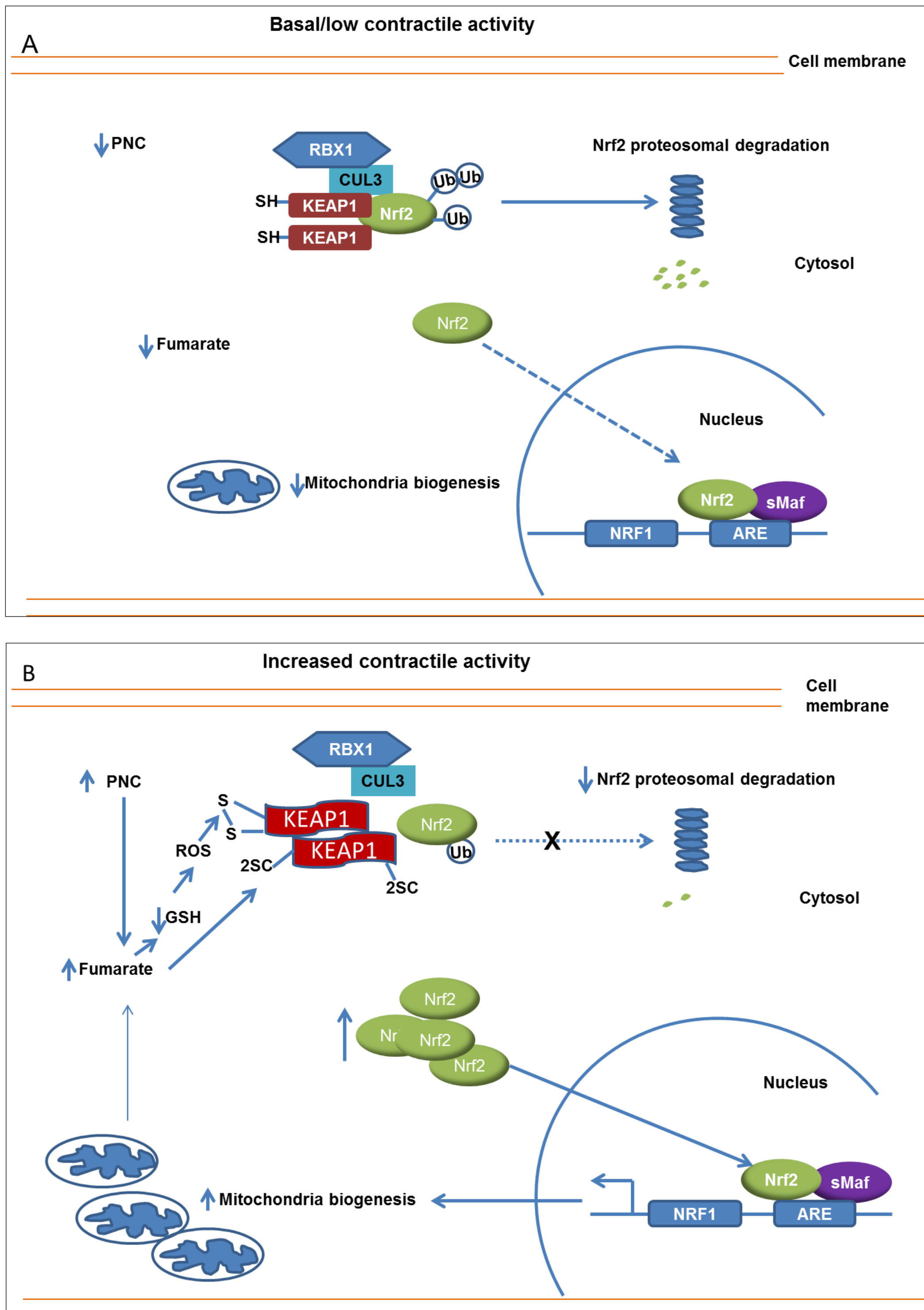

Figure 1: Illustration of putative fumarate-Nrf2-NRF1 axis in mediating exercise-induced mitochondrial biogenesis

A) Under conditions of reduced muscle contractile activity, Keap1 facilitates CUL3-RBX1-mediated targeting of Nrf2 for proteosomal degradation, leading to reduced expression of ARE-containing Nrf2-target genes. **B)** Upon marked stimulation of muscle contraction, the purine nucleotide cycle is activated leading to increased generation of fumarate in the cytosol. Marked expansion of TCAI as a result of amino acid catabolism and probably inhibition of NAD^+-dependent dehydrogenases could also lead to increased export of mitochondrial fumarate to the cytosol. Exposure of reactive Keap1 -SH groups to high cytosolic fumarate results in the formation of S-(2-succinyl) cysteine (2SC) adducts and conformational changes in its structure. This in turn diminishes Keap1's ability to facilitate CUL3-RBX1-mediated targeting of Nrf2 for proteosomal degradation. Consequently, the Nrf2 protein stabilizes, translocate into the nucleus where upon forming an heterodimer with one of the small Maf proteins activates ARE-driven expression of NRF1, leading to induction of mitochondrial biogenesis.

It is important to note that NRF1 is also activated by peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α), a master transcriptional regulator that is widely accepted to mediate exercise-induced mitochondrial biogenesis in skeletal muscles. Currently, however, there is no evidence on whether or not PGC-1α is required for Nrf2 to induce NRF1-mediated mitochondrial biogenesis. Fumarate or Nrf2 are also not known to directly or indirectly activate PGC-1α. Most likely therefore, the suggested fumarate-Nrf2-NRF1 axis might mediate contraction-induced mitochondrial biogenesis independent of the PGC-1α. Interestingly, this could account for the observation that exercise-induced mitochondrial biogenesis precedes the induction of PGC-1α expression. In one study for example, exercise was shown to

promote binding of NRF1 to the cytochrome c promoter prior to the any induction of PGC-1α expression. The cytochrome c, δ-aminolevulinate synthase and citrate synthase mRNAs levels were also found to increase prior to induction of PGC-1α protein expression. In an attempt to account for these observations, it was suggested that upon exercise the pre-existing PGC-1α molecules might initially activate mitochondrial biogenesis, which is then subsequently sustained by the newly induced PGC-1α [60]. However, this might not be the case since PGC-1α knockout mice have also been reported to exhibit robust induction of mitochondrial biogenesis and other adaptations when subjected to more less the same endurance exercise regime [61, 62]. Notably, contraction induced increase in fumarate is likely to cause rapid activation of Nrf2 signalling via Keap1 succination, which in turn could rapidly induce NRF1. This Nrf2-induced NRF1 could in turn activate mitochondrial biogenesis prior to activation of PGC-1α expression. The exercise-induced mitochondrial biogenesis observed to occur prior to induction of PGC-1α [60] might thus be mediated by the fumarate-Nrf2-NRF1 axis. Similarly, in complete absence of PGC-1α activation as in the case of PGC-1α knockout mice [61, 62] exercise could still induce mitochondrial biogenesis via fumarate-Nrf2-NRF1 axis.

Perspectives

Regular endurance type exercise regimes have long been known to be more effective in inducing adaptive mitochondrial biogenesis in skeletal muscles, compared to short resistance exercise regimes. Expectedly, prolonged endurance type exercise is more likely to activate this putative fumarate-Nrf2-NRF1 axis than short bouts of intense exercise. This is because during prolonged endurance exercise, the overall exposure of Keap1 to elevated fumarate levels will be higher (i.e larger area under the curve) compared to brief bouts of intense physical activity. The frequency of increased contractile activity might also be important factor. This is because more frequent/chronic stimulation of contraction is likely to attain a high steady state increase of intramuscular fumarate that can effectively activate the Nrf2-NRF1 axis.

Contraction-induced increase in skeletal muscle mitochondria and oxidative capacity not only enhances muscle performance, but also improves whole body metabolism. Therefore, activation of the putative signaling pathways through which contraction induces mitochondrial biogenesis is a potential strategy to enhance muscle mitochondrial content, physical performance and prevent metabolic disorders. Recently, treatment of mice with clinically relevant doses of dimethyl fumarate (DMF), a cell-permeable fumarate analogue was shown to significantly induce mitochondrial biogenesis in skeletal muscles, mainly via activation of Nrf2 signalling [63]. This indicates that accumulation of endogenous intramuscular fumarate to levels that can activate Nrf2 might indeed mediate mitochondrial biogenesis in skeletal muscles as postulated above. Therefore, besides fumarate analogues like DMF, induction of intramuscular fumarate accumulation might also be another potential strategy to induce mitochondrial biogenesis in skeletal muscles. One approach to achieve this would be activation of PNC cycle to increase fumarate generation i.e mimicking exactly what occurs during strenuous exercise. Currently, PNC activators are generally lacking but some potent small molecules activators of adenylosuccinate lyase have recently been identified, indicating that pharmacological activation of the PNC is feasible in future.

Another approach of inducing intramuscular fumarate accumulation is mild inhibition of ETC complex I. This is because inhibition of complex I would impairs oxidation of NADH leading to low mitochondrial NAD^+ levels. This in turn may impair the NAD^+-dependent dehydrogenases within the TCA cycle, including malate dehydrogenase, leading to build up of mitochondrial fumarate and other TCAI [29, 30]. Since, fumarate can be transported across mitochondrial membranes [64], cytosolic proteins like Keap1 would also be exposed to high fumarate secondary to complex I inhibition. Notably, already available drugs like metformin which are known inhibit ETC complex I have been shown to activate Nrf2 [17] and also enhances mitochondrial biogenesis. Predictably, such complex I inhibitors in combination with PNC activators would induce a more robust accumulation of fumarate in skeletal muscles.

Recent studies in some mammalian cells like activated macrophages also indicate that TCA can intrinsically be remodelled to enhance generation and release of TCAI such as succinate and fumarate for the purposes of modulating intracellular signalling and destruction of invading pathogens [44, 65, 66]. Whether this can also occur in muscle cells is unclear, but nevertheless the elucidation of the mechanism behind such TCA remodelling in macrophages might provide clues on additional potential ways to promote fumarate accumulation and subsequent activation of Nrf2-dependent mitochondrial biogenesis. The Nrf2 activators such as sulforaphane (SFN), tetrahydroxyquinone (tBHQ) and dihdro-CDDO-trifluororethyl amide (Dh404) have already been identified and reported to effectively increase Nrf2 activity in animal models should also be tested for induction of mitochondrial biogenesis in muscles [67-70].

The skeletal muscle intramyofibrillar (IMF) and subsarcolemmal (SS) mitochondrial sub-populations have long been known to respond differently to changes in contractile activity. Upon chronic increase in contractile activity, the mass and oxidative capacity of the SS mitochondria is preferentially enhanced in comparison to the IMF mitochondria [71-73]. On the other hand a decrease in muscle contractile activity leads to preferential decrease in the SS mitochondrial mass and function with very minimal effects on the IMF mitochondrial sub-population. The exact reason (s) behind this preferential alteration of SS mitochondria sub-population mass and function remain unclear. One possibility is the signalling pathways modulated by muscle contraction have preferentially effects on the biogenesis of SS mitochondrial sub-population. Notably, the skeletal muscles of patients with type 2 diabetes mellitus (T2DM) and obesity also exhibit a preferential decrease of SS mitochondrial density and oxidative capacity that can be partly rescued through regular exercise [74]. Several studies have also reported decreased activity of Nrf2 signalling in T2DM [26, 75-78]. Based on these observations, it can be suggested that the putative fumarate-Nrf2-NRF1 pathway might preferentially drive the biogenesis of the SS mitochondria. In other words, contraction-induced increase in intramuscular fumarate levels is likely to preferentially increase the SS mitochondria. The converse might be true, that is reduced contractile activity and therefore low intramuscular fumarate would cause a preferential decrease of SS mitochondria.

Interestingly, [79] observed that skeletal muscle SS mitochondria have a higher succinate dehydrogenase (SDH) activity compared to IMF mitochondria sub-population. SDH is the enzyme that generates fumarate from succinate. The SS mitochondria have also been shown to release more H_2O_2 per unit of O_2 consumed [80], but releases less amount of apoptosis inducing factor and cytochrome c in response to increased H_2O_2 when compared to IMF mitochondria [81]. These suggest that SS mitochondria metabolism seems to be inherently configured to activate Nrf2 signalling either through fumarate or ROS-dependent inhibition of Keap1. If this is the case, then the SS mitochondria population might auto-regulate its biogenesis through fumarate and/or ROS dependent modulation of Nrf2 signalling. This possibility raises an interesting perspective regarding the ragged red fiber (RRF) phenotype that is usually observed in some congenital mitochondrial myopathies due to ETC defects.

In such mitochondrial myopathies the ETC defects has been suggested to induce compensatory mitochondrial biogenesis. However, the exact reason for preferential accumulation of abnormal SS mitochondria with characteristically increased SDH activity (RRF phenotype) is unclear. Chronic ETC defect is likely to impair the NAD^+-dependent TCA cycle

reactions. The affected myofibers might also rely on the adenylate kinase reaction and PNC pathway to sustain ATP levels particularly when glucose supply is limiting. These would in turn promote intracellular fumarate accumulation. Intracellular fumarate accumulation acting through Keap1 succination, would activate Nrf2-driven biogenesis program, which might favour preferential expansion of the SS mitochondria pool with higher SDH activity. This higher SDH activity would further contribute to increased fumarate accumulation and a stronger activation of Nrf2, thus creating a vicious cycle that can lead to abnormal accumulation of SS mitochondria. In support of this possibility, mitochondrial distress due to glucotoxicity has been associated with increased intracellular fumarate levels with resultant succination of various suceptible proteins [37, 39, 82, 83]. Increased protein succination, which is indicative of increased intracellular fumarate, was also demonstrated in the brainstem of Ndufs4 knockout (Ndufs4 KO) mouse model of Leigh syndrome [84]. However, Ndufs4 KO) mouse model did not exhibit succination of skeletal muscle proteins [84], suggesting that the effects Ndufs4 KO might not have sufficiently altered the skeletal muscle fumarate levels. This might be due to tissues specific effects Ndufs4 KO as demonstrated for example by the presence of brain stem neurodegeneration but lack of myopathy in the same Ndufs4 KO mice. Recently also, activation of Nrf2 signalling has been reported in the heart of complex IV deficient Surf1−/− mice [85]. Therefore it would be interesting to investigate fumarate levels and occurrence of protein succination including Keap1 succination in animal models of mitochondrial myopathy and/or muscles of mitochondrial myopathy patients, particularly those exhibiting RRF. If indeed, fumarate-Nrf2 pathway preferential regulate the biogenesis of SS mitochondria then its inhibition should reduce RRFs associated with some mitochondrial myopathy.

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